Plant Research Department annual report 2001

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Annual Report 2001

Plant Research Department

Contents:

Risø National Laboratory

Mission

To promote an innovative and environmentally sustainable technological development within the areas of energy, industrial technology and bioproduction through research, innovation and advisory services.

Vision

Risø's research shall **extend the boundaries** for the understanding of nature's processes and interactions right down to the molecular nanoscale.

The results obtained shall **set new trends** for the development of sustainable technologies within the fields of energy, industrial technology and biotechnology.

The efforts made **shall benefit** Danish society and lead to the development of new multi-billion industries.

Risø's activities in 2001 are reported in the following publications: Risø Annual Report (available in Danish and English), Risø's Annual Performance Report (Danish) and the annual progress reports of the seven research departments (English). All publications and

Plant Research Department

Abstract

The Plant Research Department integrates modern post-genomic tools to improve our understanding of plants. The aim is to develop crops with improved agronomic traits and to engineer high-value plants, which are able to meet the growth conditions of the future environment.

The department is divided into six research programmes that are linked through their individual expertise delivered to the rest of the department. Three programmes are engaged in improving the agronomic performance of plants. Genetic tools are being developed to enhance the nutrient efficiency of plants, to strengthen the ability of plants to resist fungal attack, and to optimise flowering time. Two programmes are devoted to improving the market value of plant products. Plants with enhanced nutritional value, or that contain novel renewable resources, are designed to add value to the European Agro-Industries. A sixth programme studies the effects of the future climate on plant growth, and the

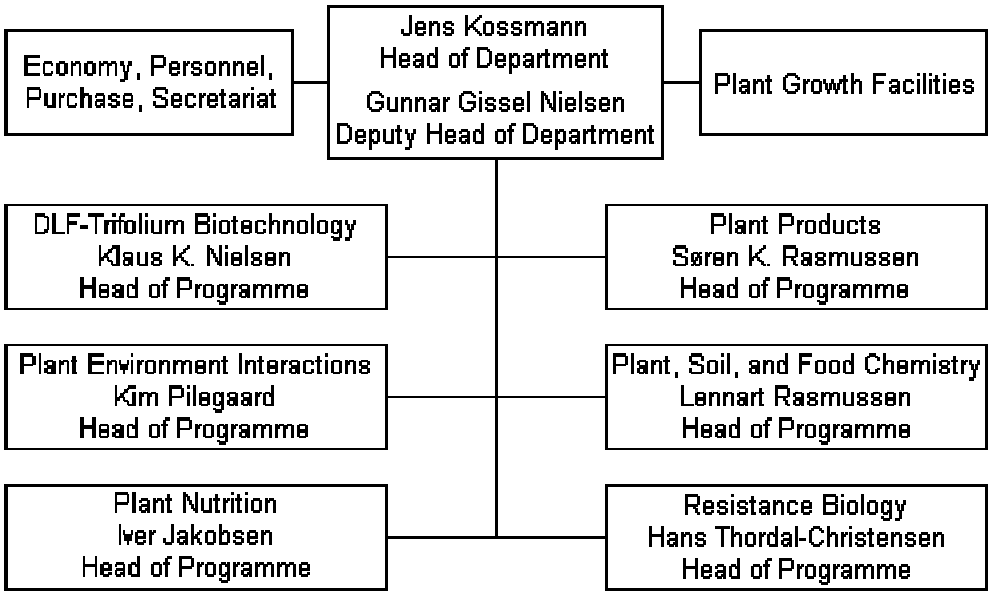
departments (English). All publications and further information can be obtained from risoe.dk. Printed publications are available from the Information Service Department, tel.: +45 4677 4004, e-mail: risoe@risoe.dk, fax: +45 4677 4013.

performance of newly designed crops and their interaction with the environment.

Diverse activities in the area of Functional Genomics integrate the department. Each programme contains special expertise in the fields of genome, transcriptome, proteome, and metabolome analyses, which are delivered throughout the department. The Plant Research Department is unique in that these activities are supplemented with a broad expertise in environmental analysis, allowing the interpretation of large biological data sets in the context of factors affecting plant growth.

http://www.risoe.dk/prd/
E-mail: prd@risoe.dk

Plant Research Department 2001



DLF-Risø Biotechnology Programme

Objectives

The biotechnology consortium between the Danish seed company DLF-TRIFOLIUM A/S and Risoe National Laboratory, PRD, is conducting a biotech research programme with a major focus on developing technologies for the control of flowering in ryegrass. An overall objective is to engineer grasses incapable of producing stems and flowers during grassland farming. The novel high-value grass varieties will have improved fodder value due to the lack of indigestible stem tissue, and the programme will provide new technologies for the avoidance of spread of active transgenes in nature (Biological Containment).

Research fields

- Identification of key genes responsible for the switch from vegetative growth to flowering
- Gene activation systems allowing control of flowering and seed production
- Test of isolated genes in transgenic grasses
- Abiotic stress tolerance
- Digestibility
- Nutritional value (fructans)
- Establishment of a monocot model transformation system
- Transposon exon trapping lines in *Arabidopsis*
- Identification of novel specific promoters
- Identification of mutations affecting the response to vernalisation

Selected reports

- A TERMINAL FLOWER1-like gene from perennial ryegrass involved in floral transition and axillary meristem identity (31 kB)
- Engineering of high quality grasses for future sustainable agriculture (101 kB)

Contact:

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Plant Environment Interactions Programme

Objectives

The aim of the programme is to study the structure, function, processes, and dynamics of agro- and semi-natural ecosystems, and the biological interactions between crops and wild plants. The goal is to predict the function of plant ecosystems in a changing environment and to assess the ecological risks of introducing genetically modified plant in the agricultural systems.

Research fields

- Soil-plant-atmosphere interactions in relation to air pollution, global change and other environmental aspects, and ecophysiology related to nutrient cycling and stress.
- Functioning of terrestrial ecosystems and impacts of global change.
- Plant fitness, competition, and environment
- Genetic resources for crops of the future
- Ecosystem modelling. Developing and improving nutrient cycling models and ecophysiological models.
- Carbon and nitrogen turn-over. Study of important processes in the turn over of nitrogen and organic matter in terrestrial ecosystems. Utilisation of nitrogen resources in intercropping systems in organic farming.
- Gene flow between crops and wild relatives
- Risk analysis of genetically modified crops
- Development of sensors and sensor systems for site specific fertilisation. Improving the nitrogen utilisation by Precision farming.

Selected reports

- Application of stable isotopes in research on plant physiology and plant-environment interactions (106 kB)
- Dispersal of transgenes from crops to related weedy and wild plants (79 kB)
- Risø Environmental Risk Assessment Facility (RERAF) (114 kB)

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Plant Nutrition Programme

Objectives

The aim of the programme is to identify and characterise processes and genes, which are crucial to the efficiency of plant nutrient uptake. The role of arbuscular mycorrhizal fungi and root hairs are central topics as well as the use of non-invasive imaging technologies. Results of the research will be used to develop crop plants with a more efficient acquisition and utilisation of nutrients in order to preserve mineral and water resources and to reduce production costs.

Research fields

- Pi uptake efficiency by mycorrhizas and root hairs; use of plant mutant tools
- *In vivo* methods for imaging of structures and transport processes in mycorrhizal fungi
- Study of nutrient transporters in *Medicago truncatula* and *Lycopersicon esculentum* in relation to mycorrhizas and nutrient status
- Functional diversity of mycorrhizal fungi as influenced by symbiotic components, soil P heterogeneity and climate change
- Use of mycorrhizal fungi for the phytostabilisation of radio-contaminated environments
- Nitrogen fixation and assimilation in symbiotic pea root nodules studied by *in vivo* ^{15}N NMR spectroscopy

Selected reports

- Symbiotic nitrogen fixation in pea root nodules studied by *in vivo* NMR (74 kB)
- The occurrence of intraspecific functional diversity in arbuscular mycorrhizal fungi (121 kB)

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Plant Products Programme

Objectives

The programme seeks to use modern technology to explore the inherited plasticity of plant organs to produce high value compounds and continuing improvement of quality and value of crop plants. Technologies are developed to convert a larger amount of natural products to compounds, which is more applicable for industrial use (waste-to-value). Metabolic engineering will be used to control biosynthesis of secondary compounds and more functionality of plant polymers and storage compounds. Research relevant to developing countries on finger millet and rice improvement is carried out.

Research fields

- Plant mitochondria with emphasis on plant-specific enzymes in the respiratory chain.
- Proteome of the the rice mitochondrial proteome with focus on phospho-proteins
- Plants response to biotic and abiotic stress, production of reactive oxygen species - ROS - and its detoxification in mitochondria and other compartments.
- Plant fibres for new biodegradable materials, identification of high value products and exploring polylactate for biopackaging.
- Pretreatment by wet-oxidation of biomass for industrial production of bioethanol.
- Waste-to-value innovation of Agro-Industrial plant products.
- Phosphate uptake and grain phytate metabolism in barley, rice and wheat.
- Exploring mutational breeding and plant transformation for new grain quality traits.
- Cereal seed and endosperm development and nutrient loading and unloading.
- Serine proteinase inhibitors involved in defence and control of regulatory pathways.
- Identification and analysis toxins in feed and food of natural and of fungal origin

Facilities

Steam explosion, pilot and loop-autoclave, chemical inert-HPLC, GC, GC-MS, FPLC and Ækta-Explorer, Multiphor II 2D IEF, ABI310 DNA-sequencer, Applied Photophysic stop-flow spectrometry, TOC-analysis.

Selected reports

- Bio refinery – production of ethanol and biogas (38 kB)
- Unexpected function of a stress-induced barley peroxidase (44 kB)

Contact

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Plant, Soil, and Food Chemistry Programme

Objectives

The aim of the research programme is to study the occurrence, transport, turnover and effects of trace elements and organic micro pollutants i agricultural and forest ecosystems and in the human food chain. Major emphasis is placed on the development of new methods and processes which can form the basis of an environmental and sustainable plant production.

Research fields

- Trace elements in agricultural and horticultural products
    - trace element profiles
    - isotope ratios
    - chemometrics
    - sediment chemistry and dating
- Organic micro contaminants in plant production
    - organic waste
    - LAS, DEHP, chlorobenzenes, and alkyl phenolic compounds
    - oil and gasoline components
    - occurrence and degradation processes for N-PAC and PAH
    - availability and plant uptake of PAH
    - natural toxines
- Air pollution and climate change
    - atmospheric chemistry and processes
    - effects of air pollutants on plants
    - effects of climate change on terrestrial ecosystems
- Pure and Applied Chemistry
- Natural Plant Protection Against Pathogenic Fungi

Selected reports

- Degradation and plant uptake of organic contaminants (155 kB)

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Resistance Biology Programme

Objective

This new Programme, which was created in the re-organisation of The Plant Research Department in the spring of 2001, performs research on a number of phenomena related to disease resistance in order to provide short and long-term solutions for controlling the damaging effects of fungal plant pathogens. The fact that plants have a natural ability to combat microbial invaders is interesting for practical as well as for scientific reasons. Genetically determined resistance has been exploited intensely in plant production, and in several ways, this is an appealing means of controlling pathogens: compared to other methods it is inexpensive, safe, and - for the growers – it is unproblematic. In addition, in third-world countries a better use of resistance contributes to a higher stability in crop production. On the back-side of the coin, however, some problems occur. Resistance cannot be used to control all pathogens, resistance can be "broken", and the breeders constantly have to look for new genetic resources of resistance. The significance of these problems has obtained a new focus at Risø with the establishment of the Resistance Biology Programme, which consists of 25-30 people.

Research fields

- Molecular biology and genetics of the barley powdery mildew fungus (*Blumeria graminis* f.sp. *hordei*)
- Genetics of resistance to *Blumeria graminis* f.sp. *hordei*
- Cellular and molecular mechanisms of resistance to *Blumeria graminis* f.sp. *hordei* in barley and *Arabidopsis thaliana*
- Genetics and cellular mechanisms of resistance to *Mycosphaerella graminicola* causing septoria tritici blotch on wheat
- Modelling of the dynamics of how pathogens spread, in particular of the wheat yellow rust fungus (*Puccinia striiformis* f.sp. *tritici*)
- Diagnostics of *Tilletia caries*, the wheat bunt fungus
- Proteomics in the Rhizobium-legume symbiosis
- DNA-marker based selection systems
- Crop improvement by means of biotechnology in developing countries
- Genetic resources for crops of the future

Selected reports

- Fungal spore dispersal (131 kB)
- Resistance in winter wheat to septoria tritici blotch (47 kB)
- Seven avirulence genes mapped in the genome of the powdery mildew fungus (39 kB)

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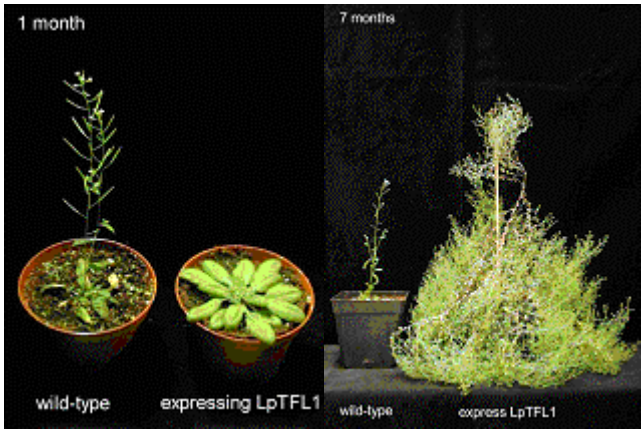
A TERMINAL FLOWER1-like gene from perennial ryegrass involved in floral transition and axillary meristem identity

Christian Sig Jensen
DLF-Risø Biotechnology Programme

Control of flowering and the regulation of plant architecture have been thoroughly investigated in a number of well-studied dicot plants such as Arabidopsis, Antirrhinum, and tobacco. However, in many important monocot seed crops, molecular information on plant reproduction is still limited. To investigate the regulation of meristem identity and the control of floral transition in perennial ryegrass (Lolium perenne) we isolated a ryegrass TERMINAL FLOWER1-like gene, LpTFL1, and characterized it for its function in ryegrass flower development. Perennial ryegrass requires a cold treatment of at least 12 weeks to induce flowering. During this period a decrease in LpTFL1 message was detected in the ryegrass apex. However, upon subsequent induction with elevated temperatures and long-day photoperiods, LpTFL1 message levels increased and reached a maximum when the ryegrass apex has formed visible spikelets. Arabidopsis plants overexpressing LpTFL1 were significantly delayed in flowering and exhibited dramatic changes in architecture such as extensive lateral branching, increased growth of all vegetative organs, and a highly increased trichome production. Furthermore, overexpression of LpTFL1 was able to complement the phenotype of the severe tfl1-14 mutant of Arabidopsis. Analysis of the LpTFL1 promoter fused to the UidA gene in Arabidopsis revealed that the promoter is active in axillary meristems, but not the apical meristem. Therefore, we suggest that LpTFL1 is a repressor of flowering and a controller of axillary meristem identity in ryegrass.

(Jensen *et al*., 2001: Plant Physiol 125(3):1517-28



Engineering of high quality grasses for future sustainable agriculture

Klaus K. Nielsen
DLF-Risø Biotechnology Programme
Keywords: Perennial ryegrass, *Lolium perenne*, non-flowering, digestibility, nutritional value.

Objectives
The biotechnology consortium between the Danish seed company DLF-TRIFOLIUM A/S and Risoe National Laboratory, PRD, is conducting a biotech research programme with a major focus on developing technologies for the control of flowering in ryegrass. An overall objective is to engineer grasses incapable of producing stems and flowers during grassland farming. The novel high-value grass varieties will have improved fodder value due to the lack of indigestible stem tissue, and the programme will provide new technologies for the avoidance of spread of active transgenes in nature (Biological Containment). Research activities include the identification of key genes responsible for the switch from vegetative growth to flowering as well as gene activation systems allowing control of flowering and seed production. A high number of genes involved in flowering have been isolated, and these are now being tested in transgenic grasses.

Background
Compared to other agricultural crops, grasses have a very positive environmental profile by being perennial, facilitating reduced mechanical treatment and very limited seepage of nutrients. Also, the use of pesticides is very limited and a large diversity of wild plant species, insects and animals thrive well in grass fields. Taken as a whole, the grass fields have a far more positive impact on the environment compared to the production of cereal-based alternatives. Therefore forage grass has a strong potential to become an important crop in future sustainable agriculture. However, the value of grass as forage is limited by the fact that especially the grass stems contain high amounts of low digestible compounds, mainly lignins and cell wall compounds coupled to lignin. As a cow can only digest a limited amount of grass the nutritional value must be improved in order to make grass account for a larger portion of the daily nutritional needs. A major breakthrough would be the development of ryegrass varieties that remain vegetated, without stem and flower tissue. The stem and flowerless ryegrass will mean a significant cost reduction due to a reduced need for more costly cereal-based concentrates. Furthermore, high quality grass will be available throughout the season, meaning more flexible and simple grazing and rotation systems. Whereas this cannot be accomplished by conventional breeding, advances in biotechnology have made it possible to transform monocot crop species, including ryegrass. This in combination with the extensive studies addressing the genetic

basis for stem and flower development in other plant species now makes it possible to engineer non-flowering ryegrass varieties.

Research fields

The transition to reproductive growth (flowering) depends upon the competence to respond to environmental stimuli and to initiate floral development. In the case of perennial ryegrass, winter conditions with cold and short days (primary induction) followed by spring conditions with increased temperature and long days (secondary induction) are required. During the primary induction there is no observable morphological development. However, a whole set of genes is at this stage either activated or inactivated as a prerequisite for the floral transition. Activated genes promote flowering, whereas inactivated genes work against flowering.

Once the relevant key regulatory genes have been isolated, the expression levels of these specific genes can be manipulated in transformed plants and, consequently, the transition to flowering inhibited. At present, a high number of up- or down-regulated genes have been isolated, including genes showing homology to well-known flowering genes such as AP1, AGL, LFY, TFL, and ID1, as well as a number of novel genes. Ryegrass plants with "knock out" or over-expression of these genes is being produced and subsequently evaluated for changes in flowering time and ability.

An alternative strategy involves the ablation of reproductive tissues through the action of toxic genes controlled by tissue and developmental specific promoters. The activity of candidate promoters is characterised by Real Time PCR, and transient- and stable ryegrass transformation of promoter::reporter fusion constructs.

In order to allow seed production, the genes used to repress flowering will be combined with an inducible expression system, in which the inhibitory action of the genes can be controlled.

The Biological Containment provided by the "non-flowering system" allows growing grasses with a wide range of genetically improved qualitative traits. Research activities initiated by the consortium include work on abiotic stress tolerance, digestibility, and nutritional value.

Another recent activity is the establishment of a monocot transformation system to serve as a novel model system allowing a fast and efficient testing of gene effects in grasses and cereals. The grass species *Brachypodium distacyon* has been chosen for its many features not unlike *Arabidosis*, *e.g.* it has a small and simple genome, short lifecycle, and self-fertility. A transformation protocol has been established, and the testing of potential flowering genes and specific promoters from ryegrass has been initiated.

As a partner of the EU 5'th Frame Work project "EXOTIC" we have produced 2300 transposon Exon trapping lines in *Arabidopsis thaliana.* The transposon element contains a promoterless GUS gene allowing the identification of mutant phenotypes and promoter activities at the same time. We are currently investigating our mutant lines for GUS expression patterns for the identification of novel specific promoters. In the near future, 20.000 Exotic Arabidopsis transposon lines will be screened for mutations affecting the response to vernalisation.

Contact

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Application of stable isotopes in research on plant physiology and plant-environment interactions

Per Ambus
Plant Environment Interactions Programme

Background

During the last decades the development of isotope ratio mass spectrometry (IRMS) has opened for new use of stable isotope methodologies in many research areas. The isotopic composition of in particular hydrogen (H), carbon (C), nitrogen (N) and oxygen (O) is very valuable as indicators of chemical and biotic processes and in the identification of origins of materials, and IRMS analysis has become a significant tool within disciplines of plant physiology and plant-environment interactions. Stable isotope work may include the experimental addition of isotopically labelled compounds as tracers, as well as the non-invasive approach of natural abundance analysis.

Many chemical and physical processes have a significant isotopic fractionation, which generally refers to an enrichment or depletion of the heavy isotope. Plants, for example, contain less ^{13}C than present in atmospheric CO_2 due to both enzymatic and physical processes during CO_2 assimilation, which discriminates against the heavier isotope ^{13}C in favour of the ligther ^{12}C. Usually the isotopic composition is expressed by the delta (δ) notation, which is a measure of the relative differences (in ‰) between isotopic ratios of the sample and an international standard.

In 1998 the Centre for Continuous Flow Stable Isotope Ratio Mass Spectrometry (CONFIRM) was installed at the Plant Environment Interactions (PLE) programme. The establishment of CONFIRM included achievement of new state-of-the-art IRMS-instrumentation suitable for natural abundance work, as well as upgrading of existing instrumentation for enriched isotopic work.

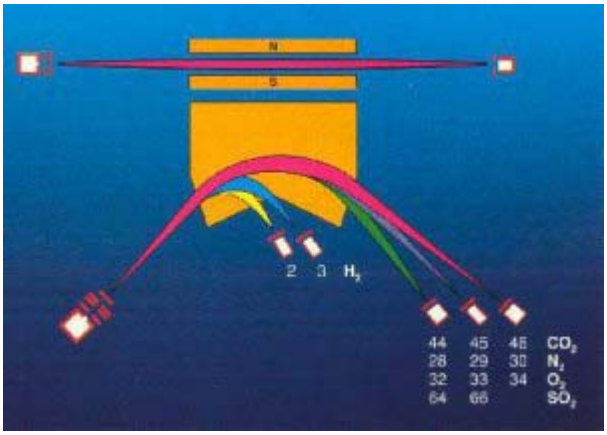

Fig. 1. Analysis of isotopic composition in light elements by magnetic separation of their ionized gaseous forms according to molecular masses.

CONFIRM has hosted several national and international joint-research projects on plant functioning and interactions with the environment. In this context, seven master students and Ph.Ds. have completed their education, and a visiting scientist from Czech Republic has been associated with CONFIRM for nine months. The facilities of CONFIRM also provide basis for isotopic analysis on commercial conditions serving both national and international costumers.

The performance and methodological capabilities of CONFIRM has been developed and improved continuously, a recent achievement being the automisation of $^{13}C/^{12}C$ and $^{18}O/^{16}O$ analysis of atmospheric CO_2.

Three case studies

Separation of root and soil respiration.

A major challenge in the understanding of C-cycling in soil-plant systems is the quantitative separation of soil-, rhizosphere- and root respiration from total soil-plant respiration. Without this knowledge our ability to predict plant-ecosystem responses to e.g. changes in climate and atmoshperic chemistry is very limited. We have performed initial experiments to examine if the CO_2 from soil and root respiration in a beech (*Fagus sylvatica*) forest could be differentiated by the $^{13}C/^{12}C$ isotopic composition.

Fig. 2. Incubation of young beech plants for the determination of ^{13}C-signatures in CO_2 from root respiration.

The $^{13}C/^{12}C$ isotopic signature, expressed as $d^{13}C$, was measured in CO_2 respired from root-free soil, in CO_2 from root respiration of young beech plants and in CO_2 emitted from the forest floor (*Fig. 2* and *Table 1*).

Table 1. Values of $\delta^{13}C$ in respired CO_2 (‰)

Root-free soil	Beech roots	Forest floor
-24.2 ± 0.4	-22.2 ± 1.0	-23.0 ± 0.4

This preliminary dataset suggests that CO_2 from root respiration may contribute up to 60 % of total belowground respiration, which is in agreement with observations in other investigations. However, the $\delta^{13}C$ values in CO_2 from the various sources are not statistically different and more effort is needed to verify the usefulnes of this approach as a tool to identify sources of CO_2.

Photorespiration and stomatal conductance

Investigations were conducted with photorespiratory mutants of barley (*Hordeum vulgare*) and *Arabidopsis*. Wild type and mutant plants deficient in the key photorespiratory enzymes glycine decarboxylase or serine hydroxymethyltransferase were grown in closed chambers with controlled CO_2 concentration, light intensity and humidity. We revealed that leaf tissue of photorespiratory mutants have lower ^{13}C content compared to the wild type plants with the difference of 5 ‰ in the atmosphere of 360 ppm CO_2 and 3.3 ‰ in 700 ppm CO_2 (*Table 2*). This difference is attributed both to the effect of stomatal conductance

on carbon isotope fractionation and the direct selection of carbon isotopes in the glycine decarboxylase reaction.

Table 2. Carbon isotope composition in leaf tisue of wild type (Maris Mink) and photorespiratory mutant (LaPr 87/30) of barley (Hordeum vulgare).

Barley type	$d^{13}C$ (‰) at 360 ppm CO_2	$d^{13}C$ (‰) at 700 ppm CO_2
Maris Mink	-27.8 ± 0.4	-26.1 ± 0.4
LaPr 87/30	-32.8 ± 0.2	-29.5 ± 0.4

The data indicate that the use of stable isotope analysis in combination with gas exchange measurements allow us to estimate the input on different physiological parameters (e.g. rate of photosynthetic carbon assimilation, stomatal conductance, photorespiration, and "dark" respiration) in adaptation of plants to changes in atmospheric CO_2 concentration.

Identification of nitrous oxide sources

Nitrous oxide (N_2O) is a powerful greenhouse gas and also influences stratospheric ozone-chemistry. In the soil rhizosphere N_2O is produced as by-products in two major biotic pathways of the N cycle, i.e. nitrification (oxidation of NH_4^+ to NO_3^-) and denitrification (reduction of NO_3^- to N_2). The fractional contribution to total N_2O production is regulated by various physical and chemical parameters in the rhizosphere and is unknown under many circumstances. Mitigation options to reduce N_2O from agroecosystems has received much attention, however, as our ability to predict N_2O production from soil-plant systems is impeded by lack of knowledge about sources, the formulation of efficacious mitigations might be unachievable.

We have investigated the sources of N_2O in grass-clover pastures, which are characterized by large nitrogen-input via biological N_2-fixation and from deposits of grazing animals, and thus posses potentials of large N_2O emissions.

Fig. 3. Grass-clover monoliths incubated for ^{15}N-labelling and detection of N_2O emissions

The source of N_2O was investigated by cross-^{15}N-labelling of the rhizosphere pools of NH_4^+ and NO_3^- combined with determinations of ^{15}N signatures of emitted N_2O (*Fig. 3*). The results show that N_2O is emitted simultaneously from both nitrification and denitrification independent of grass-clover pasture age (*Fig. 4*). The proportional contribution is seasonally dependent with nitrification being dominant in May and August and denitrification being dominant in June and October.

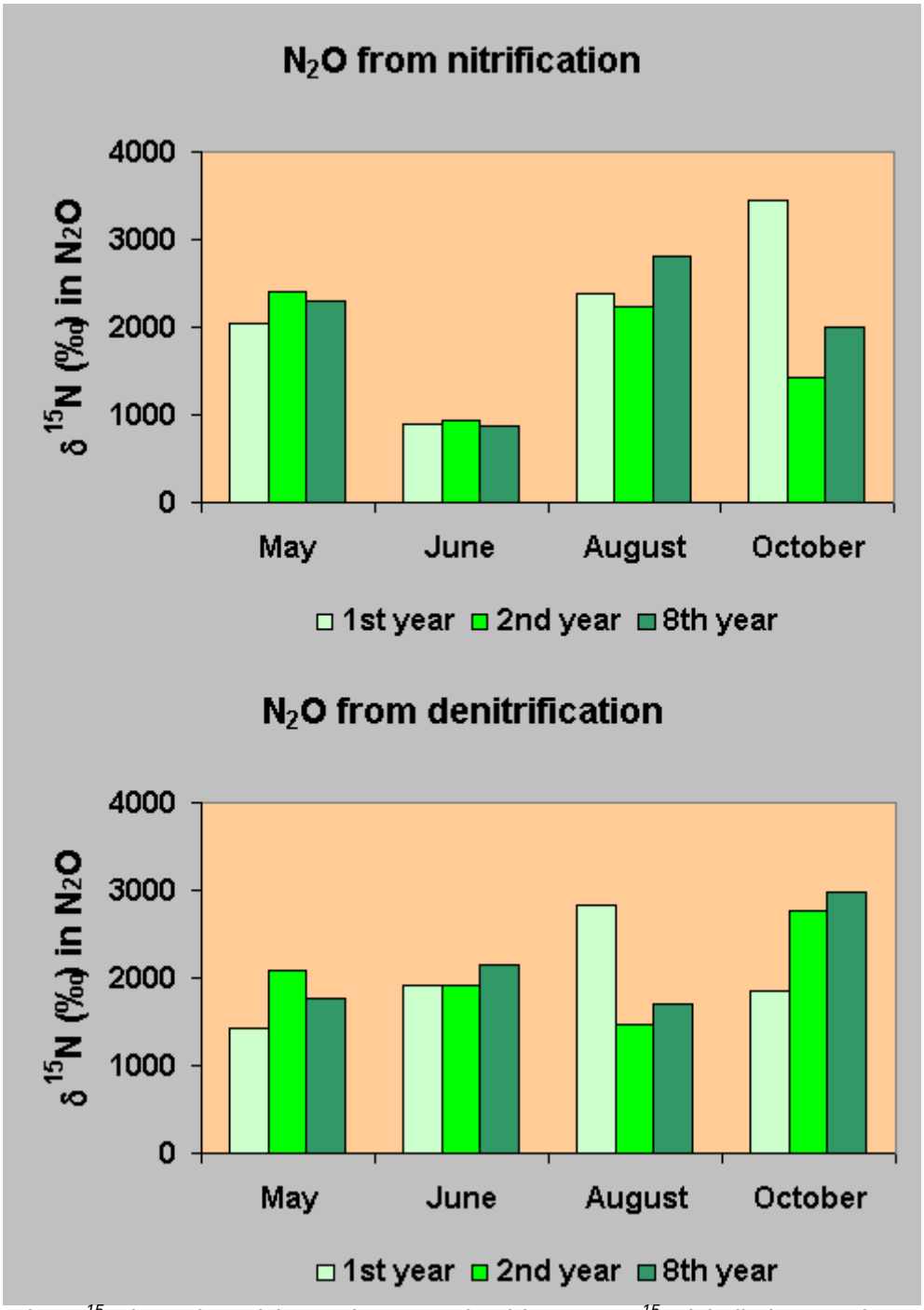

Fig. 4. ^{15}N isotopic enrichment in N_2O emitted from cross-^{15}N-labelled grass-clover monoliths of various ages.

Dispersal of transgenes from crops to related weedy and wild plants:

how does plant competition influence the process?

Thure Hauser and Rikke Bagge Jørgensen
Plant Environment Interactions Programme

Many of the world's crops are able to hybridize spontaneously with related weedy or wild species somewhere in their cultivation range. Genes may be thus be transferred to the wild species by introgression, and possibly influence their evolution and ecological performance. Among the genes may be transgenes, if genetically modified (GM) crops are cultivated in areas also hosting their wild relatives. But how likely is this? First of all the crops and wild plants should be able to hybridize. If hybrids can be formed, they have to survive and reproduce to send their genes (and among them the transgenes) onwards to new generations. Thus, the crop- and transgenes have to pass through several generations of hybrid and backcross plants to become established among the genes of the wild plants. Many different factors in the introgression process may influence whether genes are transferred and to what extent. Among these environmental conditions are known to have a large influence on the fitness of hybrids.

Oilseed rape, *Brassica napus,* and weedy *B. rapa* are able to hybridize and backcross spontaneously, and a recent study by Risø has detected introgression of cultivar genes (as opposed to only F_1 hybridization) in a weedy Danish *B. rapa* population (Hansen et al. 2001). Still, several of the processes leading to introgression are not fully understood. Former studies by Risø have shown that sometimes F_1 hybrids are more fit than *B. rapa* (Hauser et al., 1998) but sometimess less fit (Jørgensen et al. 1996), suggesting that environmental conditions may have a strong influence on hybrid fitness and the probability of gene transfer.

Among the environmental factors that we suspected could be responsible for this was plant density and frequencies of parental plants and the hybrids. We therefore studied the seed set of *B. napus*, *B. rapa*, F_1, and various backcrosses at three different densities and in several mixtures, including pure stands (Fig. 1). The study was performed in cooperation with the National Environmental Research Institute under "Center for Effects and Risks of Biotechnology in Agriculture", supported by the Danish Strategic Environmental Research Programme.

Fig. 1. Field experiment at Risø National Laboratory with different frequencies and densities of oilseed rape, B. rapa*, and various hybrid combinations*

Our results clearly show a very pronounced influence of especially the frequency of parents and hybrids on their fitness (Fig. 2):

- *B. napus*, *B. rapa* and backcross plants (F_1 ♀ x *B. rapa*) set many more seeds in pure stands than in mixtures and more seeds in stands with high frequencies of themselves.
- F_1 plants set many more seeds in mixtures and at low frequencies of itself.

Thus, F_1 hybrids produced many more seeds than *B. rapa* in mixtures, but much fewer in pure stands. Both vegetative and reproductive competition may be responsible for these effects: F_1 plants seem to be larger and more branched than especially *B. rapa* and therefore vegetatively stronger and thereby they may suppress other plants in the mixtures. Reproductive competition occurs if seeds produced by F_1 in pure plots (F_2) abort more frequently than seeds produced in mixed plots (that mostly result from backcrosses).

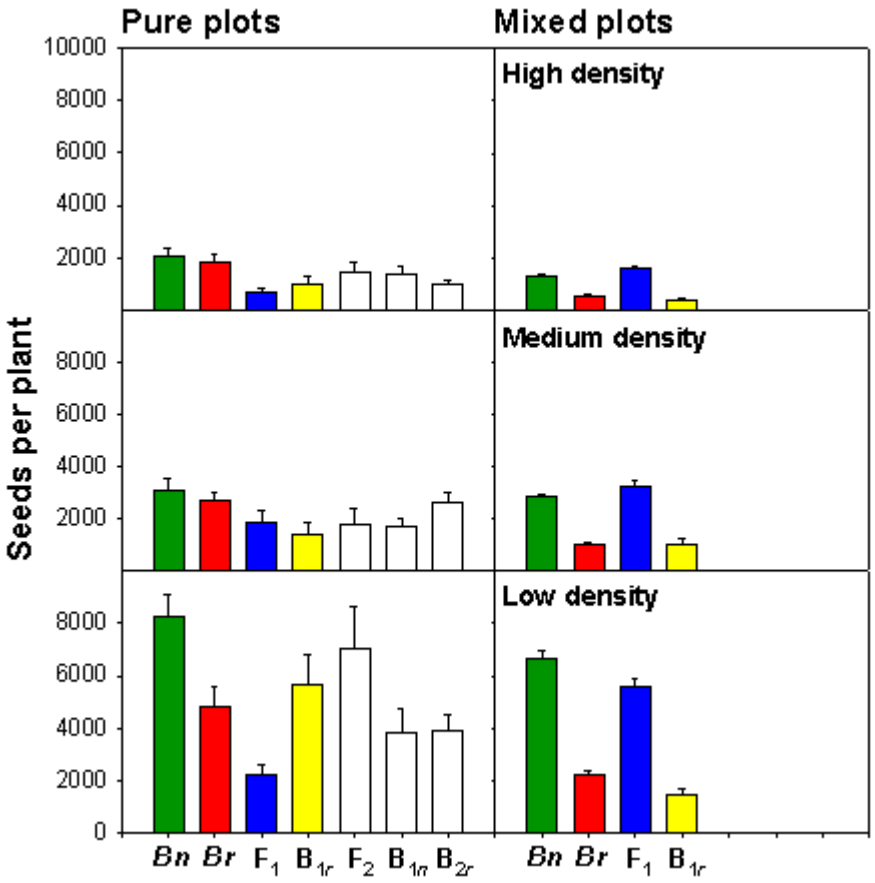

Fig. 2. Seed set of Brassica napus *(*Bn*),* B. rapa *(*Br*), and various hybrids at three different planting densities and in the pure and mixed plots (combining all the different mixtures). Backcrosses (B) have* B. rapa *(r) or* B. napus *(*n*) as the recurrent parent.*

Parallel to the experiment on seed set, a smaller study determined the paternity (the father) of seeds set by *B. rapa* in mixtures with oilseed rape and F_1 hybrids. Paternity was determined by use of characters as herbicide tolerance (the F_1 plants were tolerant), morphology, and molecular markers, and compared to an expected paternity determined by a range of fitness components studied in the field and taken from published data.

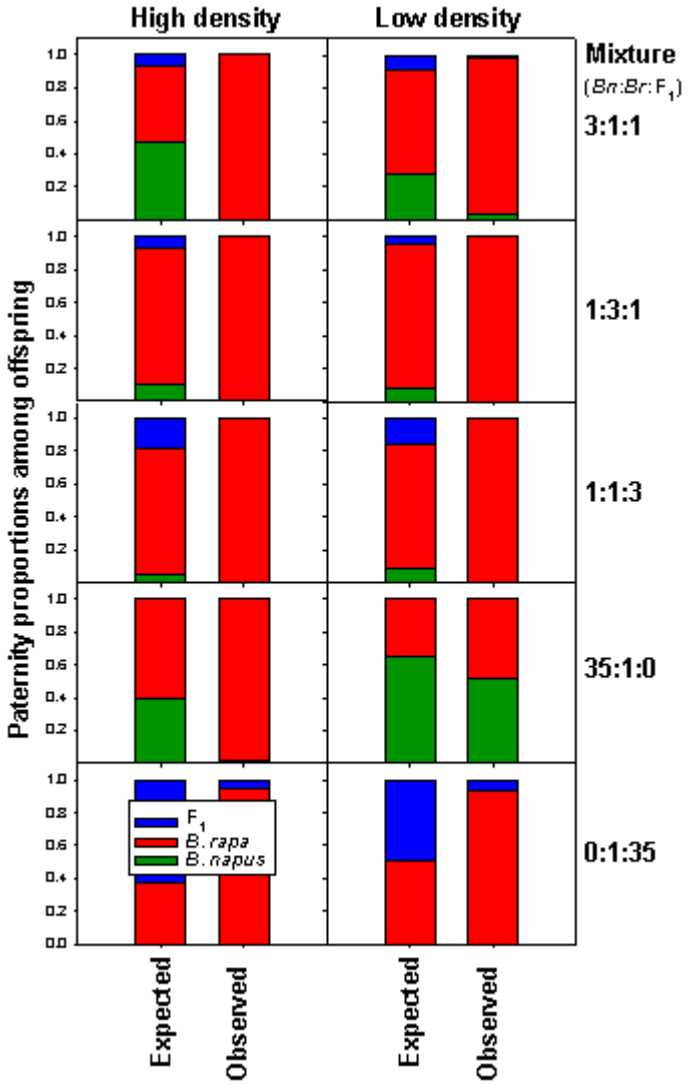

*Figure 3. Paternity proportions (*B. napus*,* B. rapa*, and F_1) expected from fitness components compared to the observed paternity proportions among* B. rapa *seeds.*

As seen in Fig 3, only relatively few F_1 and backcross offspring were found among the *B. rapa* seeds, and almost only at low density and very high frequencies of *B. napus* and F_1 fathers, respectively. Significantly fewer F_1 and backcross seeds were found than expected from the measured fitness components, showing that our understanding of the hybridization processes are still not complete.

In conclusion the two experiments show that the fitness of both oilseed rape, weedy *B. rapa*, and their hybrids may change to a large degree depending on their frequencies in the populations. They also show that even though seed set (female fitness) of F_1 hybrids (and other types of hybrids) sometimes may be rather high, they still have a very low fitness as fathers, since they only sire very few seeds through pollen. Our results point to the most likely introgression routes for transgenes: F_1 plants are most likely to transfer (trans)genes to the next generation via seeds if they occur in not too high frequencies among *B. rapa* plants, which they will frequently do in oilseed rape fields, abandoned fields etc. with many *B. rapa*. In contrast, transmission of (trans)genes from F_1 plants to *B. rapa* via pollen is much more difficult, and most likely if F_1 plants occur in much higher frequencies than *B. rapa*, which is rather unusual anywhere.

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Risø Environmental Risk Assessment Facility (RERAF)

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RERAF is a unique plant growth facility belonging to a new generation of phytotrons. Environmental risk assessment experiments can be carried out under fully controlled conditions. Plant and microbial populations, communities, and model ecosystems can be studied under climate conditions ranging from subarctic to tropical. RERAF consists today of six physically and electronically integrated, gas tight chambers, which allow exposure of plants to different air regimes.

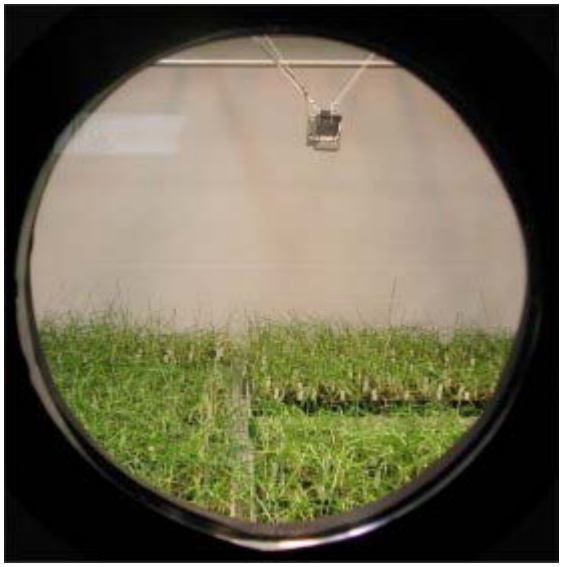

Fig.1 and 2. Different views into to a RERAF chamber.

Selected RERAF projects conducted in 2001.

Two types of projects related to global change research have been conducted in RERAF. An experiment related to enhanced concentrations of atmospheric CO_2 (Genetic resources for future agriculture) and an air temperature study (Acclimation of dark respiration).

Genetic resources for future agriculture

During the last 80 years the CO_2 concentration in the atmosphere have increased from 300 parts per million (ppm) to 370 ppm. In the next 80 years, it is predicted that the concentration will double its current level to approximately 700 ppm. These rapid changes influence the growth of plants in many aspects, but it is generally assumed that an increase in atmospheric CO_2 will increase e.g. growth and seed yield, however a RERAF study with oilseed rape previously indicated that modern sorts compared to old sorts can be poorer adapted to CO_2 changes resulting in a lower seed yield. A possible explanation for this result could be a reduction in the genetic variation due to many years of plant breeding. To investigate if this is also the case for oat a new study was conducted in RERAF. Different Scandinavian sorts of oat were exposed to two different CO_2 regimes (370 and 700ppm). Seed material was obtained from the Nordic gene bank and Danish, Norwegian and Swedish sorts developed before 1920 were compared to younger types. Some sorts were also compared in relation to interaction with a drought treatment during the growth period. The following parameters were investigated during the treatments: Chlorophyll fluorescence, gas exchange and xylem water potential. After harvest, biomass and seed yield will be determined.

Fig. 3. In RERAF the oat plants grew very well. Their height reached 2 meters.

Acclimation of dark respiration

A set-up in RERAF was used to study acclimation of respiration. There is growing evidence that plant respiration acclimates to long-term changes in growth temperature and that CO_2 exchange models need to take acclimation into account when estimating the effects of global warming on respiratory CO_2 release over long periods. Respiration is normally temperature dependent, with short-term increases in temperature resulting in exponential increases in the respiration rate over much of the temperature range. Longer-term changes in the growth temperature often result in respiratory acclimation in both leaves and roots. Acclimation occurs within a few days in many species. The mechanisms responsible for acclimation of respiration have yet to be fully elucidated and it is unknown if respiration acclimates to average temperature (day or/and night) or extreme temperatures (max. or min. during the day or/and night). To investigate this, a RERAF experiment on root and leaf respiration were conducted after 8 days of several different combinations of temperature treatments on following plant species: *Quercus, Plantago euryphylla, Plantago lanceolata, Brassica napus s.sp. napus, Crampe hispanica, Raphanus sativus, Zea mays, Sorghum bicolour.*

Our data demonstrates that plant respiration does not acclimate to one type of growth temperature across a broad range of genotypes. Moreover, it appears that the growth temperature to which respiration acclimates cannot be predicted from characteristics such as the tissue (roots versus leaves), functional group, and type of photosynthesis and/or growth rate.


Fig. 4. and 5. The different plant species used in the study and a leaf prepared for respiration measurement.

Symbiotic nitrogen fixation in pea root nodules studied by *in vivo* NMR

Anne Marie Scharff
Plant Nutrition Programme

In rhizobia-leguminous plant symbioses ammonium is generated by the action of the bacteroid enzyme nitrogenase. The current model of nitrogen transfer from the bacteroid to the plant suggests that ammonia diffuses across the bacteroid membrane and is assimilated into amino acids in the plant cytoplasm. However, the transport of symbiotically fixed nitrogen across the membranes surrounding the bacteroid and the form in which this occurs is a matter of controversy. We have investigated $^{15}N_2$ fixing pea root nodules root by *in vivo* ^{15}N NMR spectroscopy in order to obtain non-invasive information on the nitrogen fixation and assimilation processes.

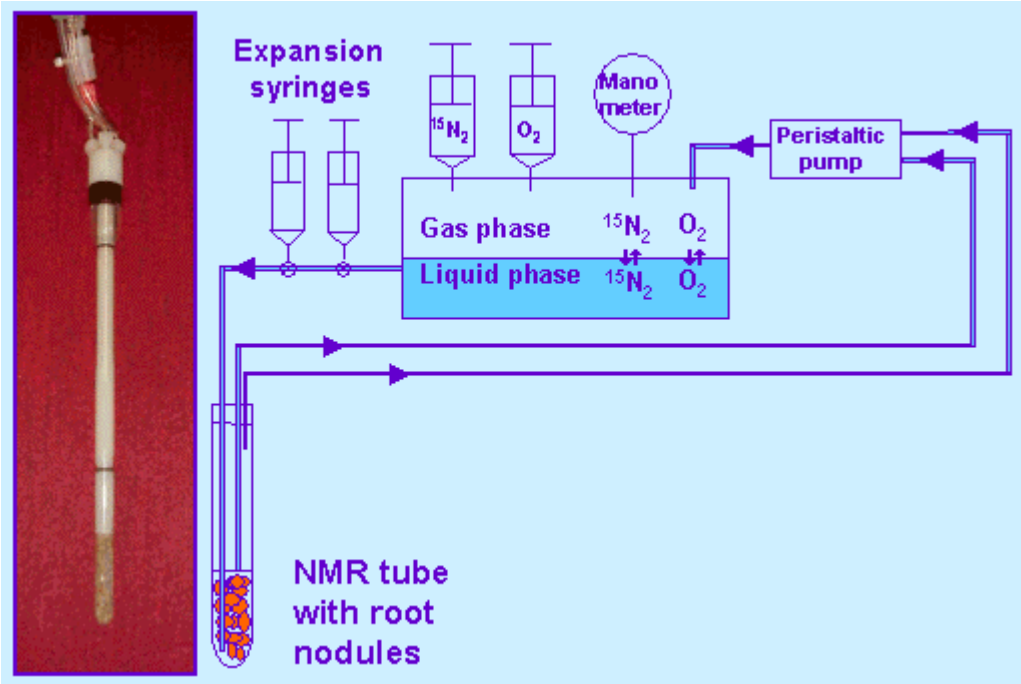

Fig. 1. Perfusion system used for studying nitrogen fixation and assimilation in living pea root nodules by ^{15}N NMR spectroscopy. Approximately 1 g FW root nodules could be contained within the detection volume of the NMR tube

We have developed a perfusion system (Fig. 1) for maintaining the pea root nodules in a physiologically viable and $^{15}N_2$ fixing state while incubated in the limited volume of the NMR tube. Especially the oxygen supply to the root nodules is a critical factor in maintaining the high oxidative phosphorylation level needed to support nitrogen fixation. *In vivo* ^{31}P NMR spectroscopy was a convenient non-invasive method for monitoring the physiological state of the nodules. A well-oxygenated tissue has a high ratio of ATP to ADP and this was the case for root nodules in the perfusion system (Fig. 2). Unchanged metabolic activity could be maintained for more than 8 hours. The chemical shift of the phosphate signal is pH-dependent and this feature makes it possible to estimate intracellular and even subcellular pH values in a living tissue. The pH of the root nodule cytoplasm was estimated to 7.2 and the vacuolar pH was approximately 5.2 (Fig. 3).

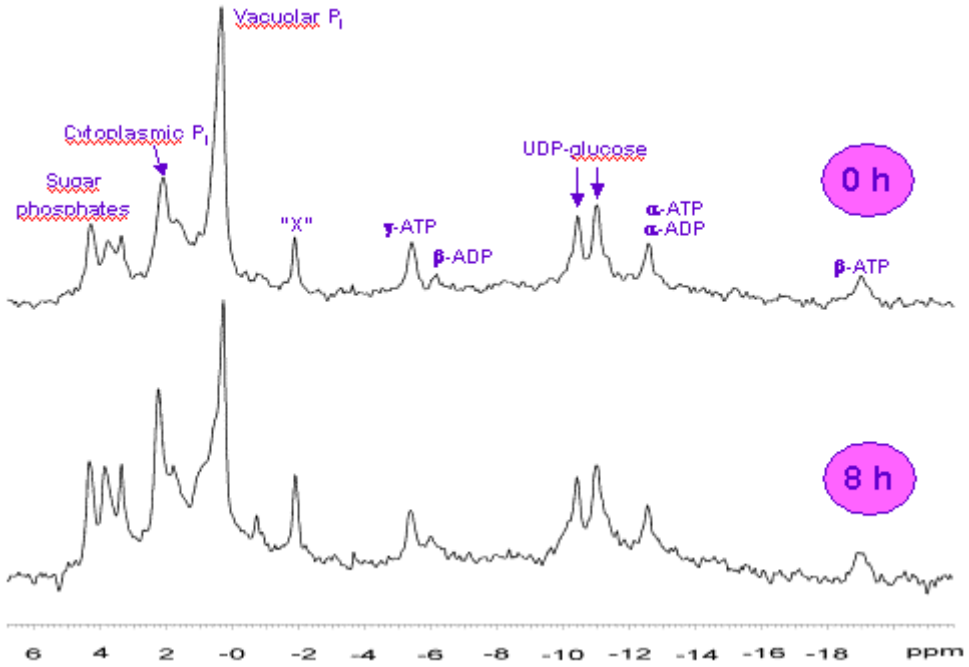

Fig. 2. In vivo ^{31}*P NMR spectra of pea root nodules showing the unchanged metabolic status during an eight-hour incubation period in the perfusion system*

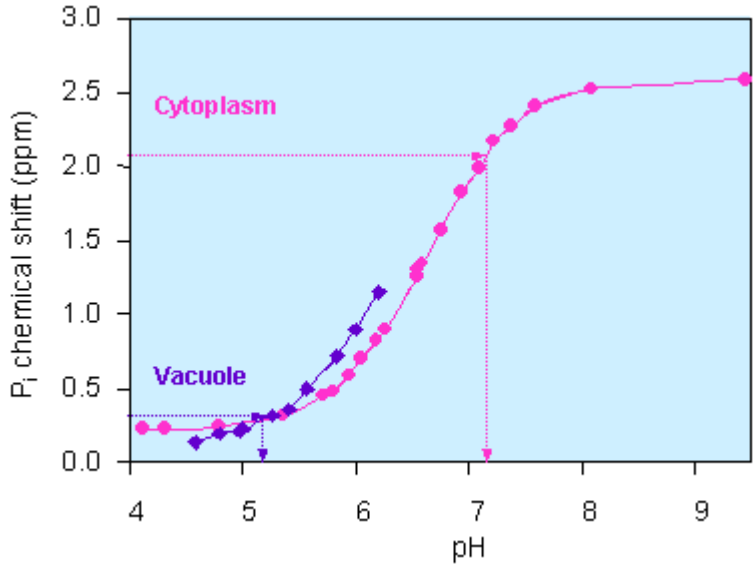

Fig. 3. The intracellular pH of the cytoplasm and the vacuoles of living pea root nodules were determined from the phosphate chemical shift

A time course of *in vivo* ^{15}N NMR spectra of $^{15}N_2$ fixing pea root nodules was recorded (Fig. 4). Previously unreported, this demonstrated that it is indeed possible to apply *in vivo* ^{15}N NMR spectroscopy to the study of nitrogen fixation and assimilation in root nodules. By using NMR it was possible to observe directly the incorporation of ^{15}N into living nodules, but detection was limited to ammonium and some of the more abundant amino acids because of the relatively low sensitivity of the method.

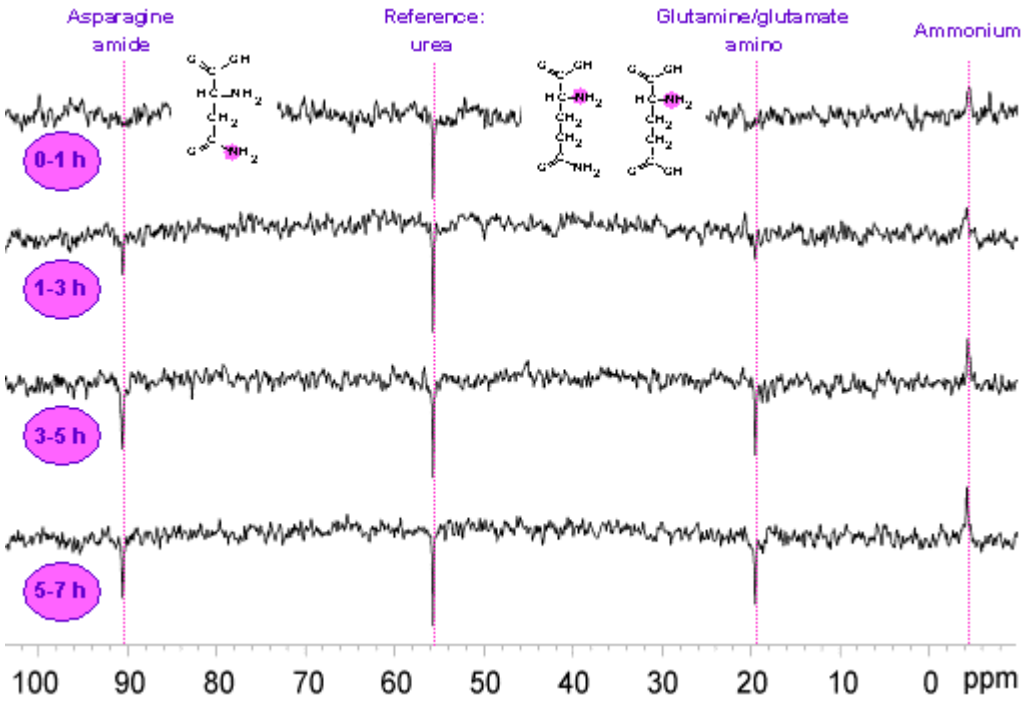

Fig. 4. In vivo ^{15}N *NMR spectra showing a time course of nitrogen assimilation in* $^{15}N_2$ *fixing pea root nodules*

A substantial pool of free ammonium was observed to be present in the metabolically active, intact symbiosis. This non-invasive observation is an important new information in the on-going discussion concerning the assimilation of fixed nitrogen in root nodules. The ammonium ions were located in an intracellular environment, which caused a remarkable change in the *in vivo* $^{15}NH_4^+$ chemical shift when compared to all other systems previously studied by *in vivo* ^{15}N NMR. Alkalinity of the ammonium-containing compartment is suggested as a possible explanation for the unusual chemical shift (cf. Fig. 5A). The observations thus point to the bacteroids as a probable location of the free ammonium pool in root nodules, as the bacteroid cytoplasm has previously been anticipated to be rather alkaline because of the proton pumping activity of the electron transport chain in the bacteroid inner membrane as well as the proton consuming nitrogenase activity (cf. Fig. 5B).

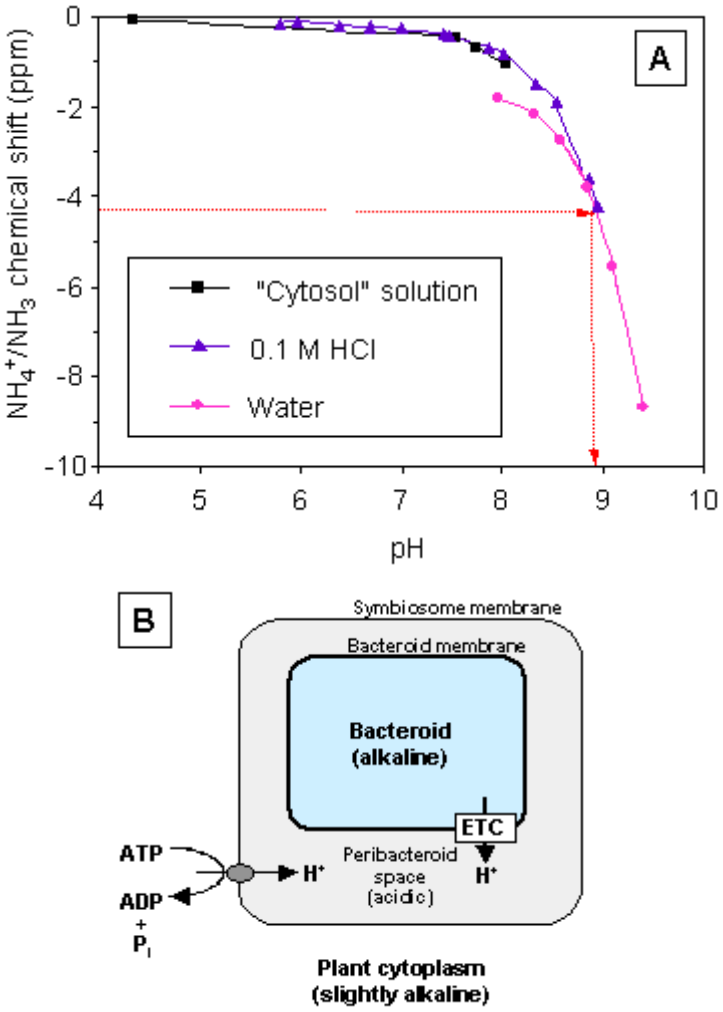

Fig. 5. (A) The unusual chemical shift of ^{15}N-ammonium would correspond to a pH of 8.9. (B) It has been suggested that the bacteroid cytoplasm is rather alkaline because of the proton pumping activity of the electron transport chain (ETC) in the bacteroid inner membrane as well as the proton consuming nitrogenase activity

The observed ^{15}N labelled amino acids, glutamine and/or glutamate, apparently reside in a different compartment, presumably the plant cytoplasm, as no changes in their expected *in vivo* ^{15}N chemical shifts were observed.

The occurrence of intraspecific functional diversity in arbuscular mycorrhizal fungi

Lisa Munkvold Knudsen
Plant Nutrition Programme

Molecular analytical tools have revealed that the genetics of arbuscular mycorrhizal fungi (AMF) is extremely complex in comparison to most other organisms. Furthermore, a functional grouping of these organisms is required in order to gain meaningful information on ecosystem functioning from a taxonomic analysis of the AMF community. It is still unclear whether a common function may be assigned at the level of nucleus, spore, isolate, species or even a cluster of species.

We studied the functional diversity of 16 *Glomus claroideum*, 13 *G. mosseae* and 6 *G. geosporum / caledonium* isolates by measuring a range of variables functional in growth and P uptake and subsequently by comparing the results to a phylogenetic analysis of the large ribosomal subunit (LSU) sequences of the isolates. All isolates were grown in low-P soil in association with cucumber (*Cucumis sativus* L.) in a cross-tube growth system with a single side arm (Fig. 1). Soil in the side arm (hyphal compartment, HC) contained radioactive phosphate (^{33}P), which was separated from the root compartment (RC) by 25µm mesh, preventing root entry, and a 1 cm soil layer containing no ^{33}P.

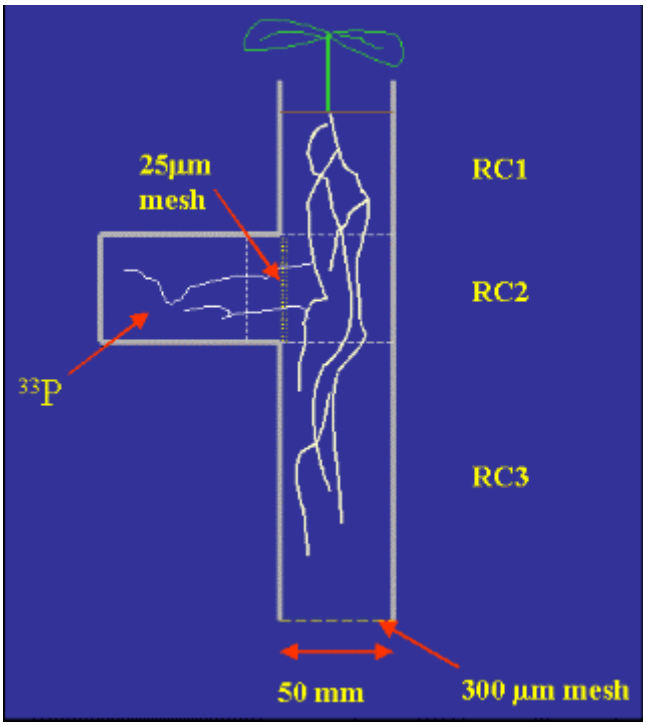

Fig. 1.Cross-tube growth system with radioactive labelled phosphorus in the hyphal compartment (side arm).

Both plant growth and P content varied considerably between isolates, but the two variables were closely correlated (R^2 = 0.85) (Fig. 2). This confirms that P uptake is a crucial functional AMF variable for explaining their ability to improve plant growth in low P soil. The variation in AMF P uptake from soil to plant could be caused by variations in either the patterns of fungal growth in roots and soil or the specific effectiveness of the AMF in P uptake.

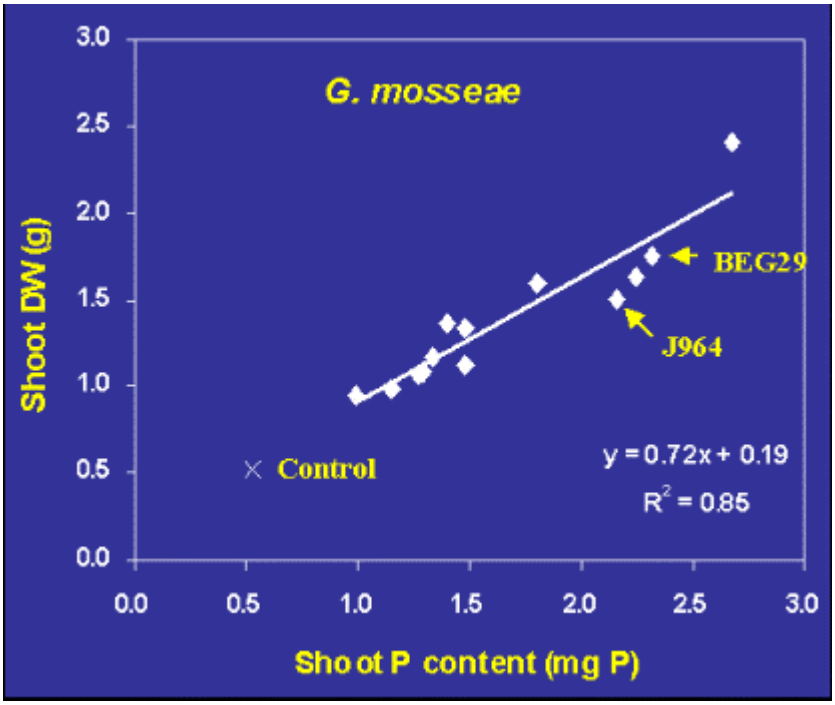

Fig. 2. The relationship between shoot growth and shoot P content in cucumber grown in association with 13 isolates of G. mosseae*.*

All AMF isolates produced well-developed mycorrhizas (Fig. 3), but the variation observed in percent root length colonised showed a poor relationship with plant P content (R^2 = 0.003). Likewise, a marked variation observed in the amount of external hyphae produced was not correlated with plant P content. However, a correlation was found between P uptake from the radiolabelled soil and its content of AMF biomass measured as hyphal length (Fig. 4). Similar correlations were observed for *G. claroideum* (R^2 = 0.62) and *G. geosporum / caledonium* (R^2 = 0.82). Phosphate uptake per unit length of AMF hyphae therefore seems to be a constant and well-conserved character within the three AMF species studied.

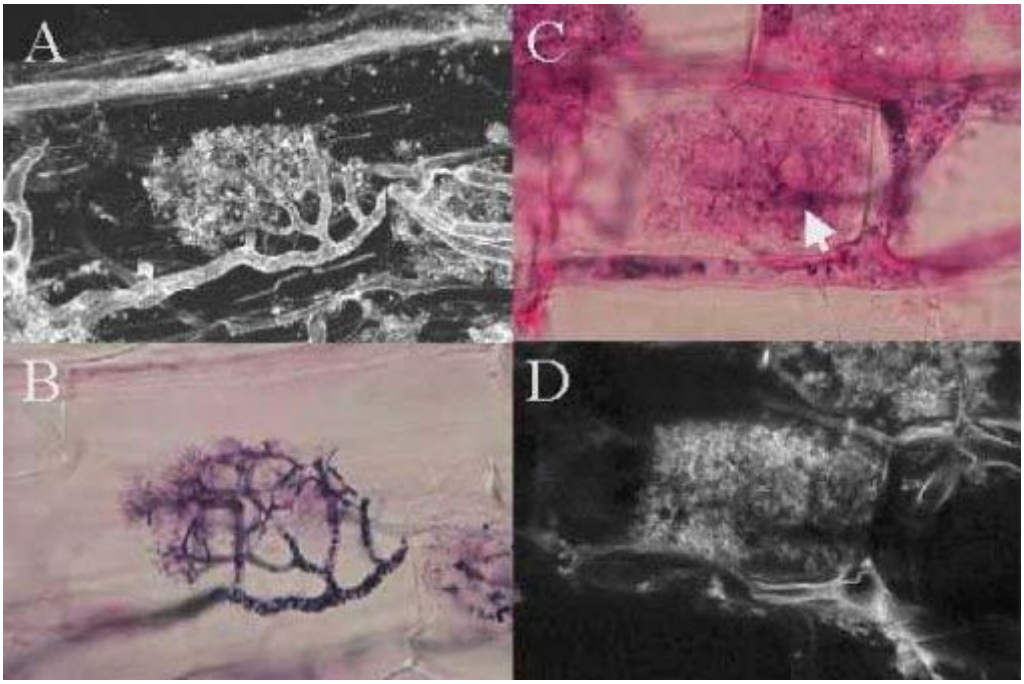

Fig. 3. Colonisation of cucumber roots by G. mosseae*. Viable parts of the fungus were visualised by staining for succinate dehydrogenase activity (purple), while the total fungal tissue was visualised by staining with acid fuchsin (pink). Picture A and B show a young forming arbuscule by laser scanning confocal microscopy (LSCM) and light microscopy*

(LM). Picture C (LM) and D (LSCM) show a dying arbuscule (pink) with an active trunk hyphae (purple, white arrow).

Two contrasting isolates, BEG29 and J964, are marked with arrows in Fig. 4. The soil hyphae of BEG29 did not spread into the radiolabelled soil, but still produced a marked response in plant growth and P content similar to that of J964. This implies that BEG29 had an efficient P uptake from rhizosphere soil, and that the uptake took place within 1 cm distance from the root surface. Our study is the first to demonstrate that growth patterns of AMF may vary considerably between isolates of one AMF species without influencing the growth-promoting effect of the fungi. This finding has significant implications for our understanding of the functioning of AMF communities: Two AMF with a contrasting spatial exploitation of soil P would seem to be highly complementary in terms of overall P uptake effectiveness.

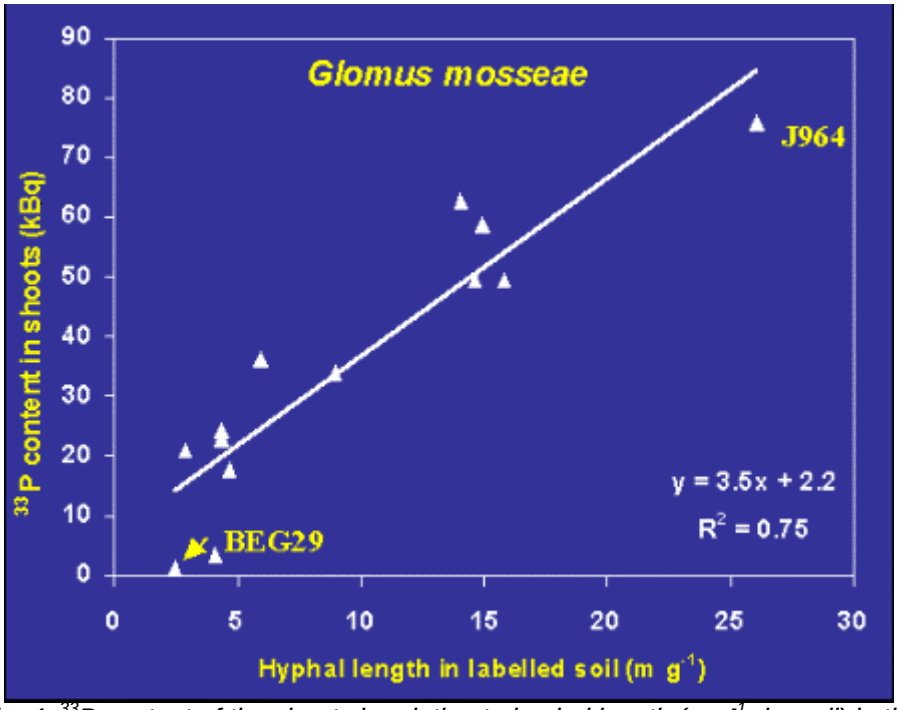

Fig. 4. ^{33}P content of the shoots in relation to hyphal length (m g^{-1} dry soil) in the radiolabelled soil (R^2= 0.75) for cucumber plants associated with 13 different G. mosseae *isolates.*

DNA encoding the internally transcribed spacer region of the large ribosomal subunit (LSU) was amplified and sequenced for some of the fungal isolates. The subsequent phylogenetic analysis, showed that isolates from *G. mosseae, G. geosporum* and *G. caledonium* formed well bootstrapped clusters, i.e. they formed clusters in respectively 71, 97 and 61 of 100 analyses. Isolate sequences differed, but the relationship between isolates could not be established (Fig. 5). LSU sequences were obviously not sufficient to suggest any genetic explanation for the observed differences in function. Functionally meaningful genetic markers thereforeneeds to be identified that might resolve the relationship between isolates more clearly. However, such marker identification is a difficult task, as the function of most sequenced AMF genes is still unknown.

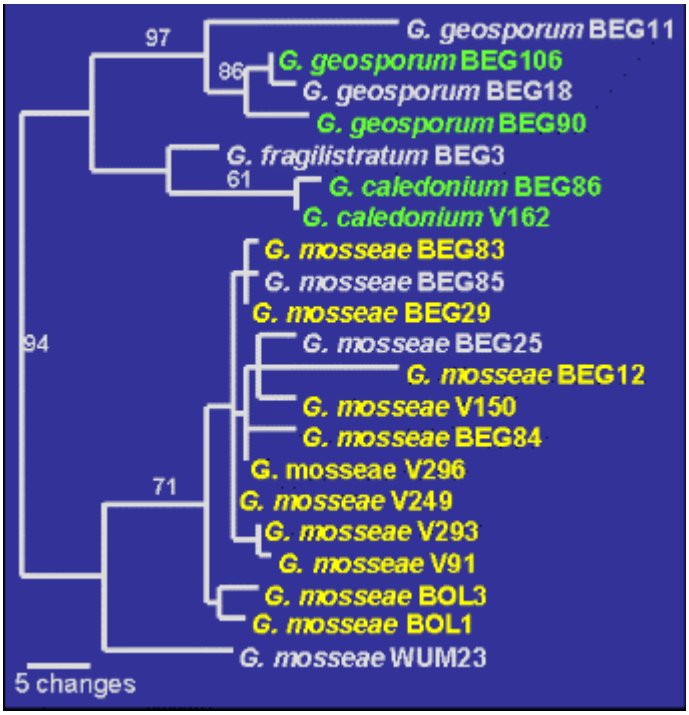

Fig. 5. Consensus tree for G. mosseae *and* G. geosporum / caledonium *isolates based on large ribosomal subunit sequences. Analyses were carried out by R. Kjøller and S. Rosendahl, Copenhagen University.*

In most previous studies, the particular AMF isolate used has received only little attention. Species were compared on the basis of results obtained with only one isolate. The present study clearly demonstrates that the result of an experiment will depend on not only the species used, but also the isolate selected. Our results also underline that extrapolation from the isolate to the species or the community level should be carried out with great caution.

Bio refinery – production of ethanol and biogas

Anne Belinda Thomsen
Plant Products Programme

Biomass is considered carbon dioxide neutral because it takes up as much of this gas under growth as it eventually will return to nature when biologically broken down or burned. To help maintain the carbon dioxide balance, one should attempt to utilize energy from plant resources in the best possible way before the biomass are combusted into carbon dioxide and water. A good way to make use of the energy in plants is to replace the non-carbon dioxide neutral fuel propellants with carbon dioxide neutral ones. By replacing gasoline, diesel oil or MTBE, with ethanol the gain is greatest if the latter can be produced from plant remains, which often have no other useful application.

MTBE, methyl-tertiary-butyl-ether, is added to gasoline in order to increase the octane number, to replace lead in gasoline. Unfortunately, this substance is both mobile and persistent in nature. It breaks down only with difficulty if, for example, it should be released into groundwater. As gasoline is stored in underground tanks, which can be in danger of leaking, MTBE has, to a rising extent, been observed in ground water resources throughout the western world, and its use has therefore been banned in California, USA from year 2002. Ethanol produced from renewable resources can replace the use of MTBE in gasoline, with no negative consequences on the environment.

Denmark's emissions of carbon dioxide must be cut down in order to meet the national goals of a reduction, relative to 1988, of 20% in 2005 and 50% in 2030 (ref.1). With respect to the carbon dioxide levels set for 2005, the present initiatives are insufficient; the emissions are to be reduced by a further 3.6%. The measures to be taken to achieve this can be to introduce energy savings in both the industrial and transport sectors. The contribution of the transport sector to the carbon dioxide emission is 20% of the total carbon dioxide production (ref.2). Replacing 10% of the gasoline consumption with a carbon dioxide neutral fuel such as ethanol, produced from primary or secondary biomass, will contribute with a saving in carbon dioxide of approximately 2%.

The Danish Bioethanol Concept
Since 1994 Risø National Laboratory and the Technical University of Denmark and have co-operated on developing new technologies to produce bioethanol from waste biomasses such as straw and wood chips - both also termed lignocellulosic materials. For the first time there has been success in utilizing straw and other raw materials to this objective, without burdening the environment and at the same time gaining a good economic result in the process. The good economy is achieved with all the carbon in the straw utilized, finding neither wastewater nor emission products resulting from the process.

Conventional biogas reactor will utilise only half of the biomass resource for the biogas production. Lignocellulosic materials pass almost unconverted through the biogas unit. In contrast, a bioethanol production plant is designed to open lignocellulosic fractions and convert the released sugars into ethanol. In this process only the saccharides are converted into ethanol, while the remaining biomass fractions traditionally are lost in the effluent or burned in a boiler. Also by-products and other compounds formed during the fermentation such as acetate are usually lost. A combination of these two processes in a production plant will have great advantages, as it would be possible to handle wider range of lignocellulosic biomass like wheat straw, grasses and wood chips together with fibres from manure. These fractions are opened using wet oxidation and the liberated sugars fermented into ethanol. The non-fibrous fraction of the manure and the residual compounds from the ethanol fermentation can subsequently be converted into methane in a biogas reactor. Experiments have shown that the introduction of a biogas step will remove the lignin derivates and other toxic compounds known to inhibit the ethanol fermenting organisms when process water is recirculated to a high degree. Another advantage is that the nutrients originally present in manure can reduce the requirements of growth factors for the micro-organism in the ethanol fermentation. The concept is still in

a preliminary state, but all individual steps in the process have been tested in laboratory scale.

In Brazil ethanol is produced for automobiles out of sugarcane, whereas in Denmark straw with its content of 70 – 80 % sugar, will serve as a good resource for the production of ethanol. But there are two essential bottlenecks in utilizing straw to produce ethanol, through a bacterial process: (1) the material acts to bind the sugar substance together thereby hindering the bacterial attack of the sugar backbone – i.e. lignin; (2) the crystalline structure in the cellulose hampers the enzymatic release of glucose. Therefore a pre-treatment is needed to modify these two effects.

In the combined bioethanol and biogas production a pre-treatment by wet oxidation is introduced. This pre-treatment breakes up and removes lignin by oxidation and at the same time the crystalline formation of the cellulose is broken down. In this way, the sugar substances, cellulose and hemicellulose, are released to enzymatic treatment and ethanol fermentation. Neither mission products nor toxic substances are released, which can hamper the ethanol fermentation. First hexoses are fermented by traditional baker's yeast, *Saccharomyces cerevisiae* into ethanol by performing the enzymatic glucose liberation and the microbial fermentation in one process step - referred to as SSF (simultaneous saccharification and fermentation) - product and substrate inhibition will be minimized, yielding higher overall ethanol productivity. Preceding the glucose fermentation, the pentoses – primarily xylose – are fermented into ethanol by a thermophilic anaerobic bacterium, *Thermoanaerobacter mathranii*.

Process wastewater contains oxidation products from the pre-treatment of lignin and by-products produces in the fermentation. These compounds from the ethanol production is a valuable resource in the following biogas reactor, rather than being a problematic waste product. The biogas formed from the process water gives added value of approximately 26 %, and therefor ethanol can be produced relatively cheaper compared commonly used processes in other countries. The biogas process leaves the water with limited organic material and enables it to be recirculated in the process. Further cleaning of any wastewater is not required, but can be utilized as fertilizer in farmlands, and contributing to a sound overall process economy.

A thorough economic evaluation of all aspects of the process from the treatment and storage of straw to the production of ethanol and biogas (methane) yields an estimate of the ethanol in the range of 2.00 to 2.75 Danish crowns per litre ethanol. It is therefore possible to compete with the price of gasoline (2.15 to 2.48 DKK) as well as the price of MTBE of 3.64 DKK. The price of ethanol produced from grain or corn stover in the USA today is between 2.10 and 2.64 DKK per litre.

What are the raw materials and how much ethanol is formed from it?
In principle, all raw materials composed of lignocellulose can be used in the process. These would include straw, wood chips, garden waste, and household waste, such as paper products, containing cellulose. The ethanol derived from different types of sugar or different raw materials can vary, also with different harvest years. As a rule of thumb, one kilogram of raw material, such as wheat straw, yields 250 grams of ethanol.

An estimate: If an ordinary house garden produces 400 kilograms of garden waste per year, this can be converted into 100 kilograms of ethanol, corresponding to 125 litres of ethanol. On the average, a Danish car drives 41 kilometres per day (Ref.1). The average fuel consumption is 12 kilometres per litre gasoline, corresponding to 3.4 litres per day or 1240 litres per year. By replacing the MTBE in gasoline with ethanol, substituting approximately 10 %, an ordinary garden will be able to supply a car with a year's ethanol consumption.

How far have we come and what are the prospects?
Currently work proceeds to get a pilot production plant established for demonstrating and optimizing a comprehensive project. This involves the investing of 30 to 40 million Danish crowns including equipment and working force. It is estimated that a pilot plant will be able to stand ready from a starting date during six months to one year and be tested throughout and adjusted during an additional year. In this way the process will be ready for commercial use. An industrial plant will be able to be built afterward within six months to a year.

Six million tons of wheat straw was produced in Denmark in 1996, of which only about 15% were used for energy purposes (Ref.3). About 1.8 million tons of straw is found to be surplus, which means that it has no direct application. If about half of this surplus were used to produce ethanol, six ethanol production plants could be supplied each with a capacity of 150,000 tons of straw per year. The combined production from these plants would be 200,000 tons of ethanol per year (together with about 120 million m^3 biogas per year). This would account for all needed ethanol if 10% of Denmark's use of gasoline is to be substituted.

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Unexpected function of a stress-induced barley peroxidase

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Plant Products Programme

We are investigating the functions of secretory plant peroxidases, which are glycosylated monomeric heme enzymes. The three dimensional structure of plant peroxidases is robust and stabilized by four intramolecular disulfide bridges and two calcium ions. Peroxidases are capable of oxidizing a wide variety of substrates under the consumption of hydrogen peroxide. This versatility makes it difficult to assess the biological function of peroxidases, but makes them interesting as industrial enzymes. The physiologically most relevant substrates are phenolic compounds, which often are derived from the core phenylpropanoid pathway. Two oxidized substrates are released as radicals for every full reaction cycle of the peroxidase. The released radicals can spontaneously react with each other to produce dimers, higher order multimers, or act toxically by attacking nearby biological membranes. These reactions take place in the maturing, fully extended plant cell wall during the process of lignification and can also be located in cell wall appositions formed in response to stresses, such as wounding or pathogen attack.

Barley plants combat the biotrophic powdery mildew fungus *Blumeria graminis* f.sp. *hordei* (*Bgh*) partly by the formation of cell wall appositions, known as papillae. Peroxidases and their substrates accumulate in developing papillae and *Bgh* penetration is thus likely to be affected by the speed and extent of peroxidase-mediated cross-linking of phenolic compounds in the papillae.

The Prx7 peroxidase accumulates in vacuoles, as revealed by immuno-gold localisation using monoclonal antibodies. This localisation was predicted from both the cDNA sequence and peptide mass analysis of the mature protein that was consistent with the cleavage of a C-terminal signal peptide. The cell wall peroxidase Prx8 does accumulate at papillae, but more strongly in cell walls below the attacked wall. We have analysed if Prx8 and Prx7 might increase the resistance of barley against *Bgh* and compared their function in the resistance response of barley cells.

Single Cell Functional Analysis
We have utilized a recently developed assay to measure the ability of the fungus to complete its lifecycle on cells expressing putatively anti-fungal genes. Genes encoding the defence-related peroxidases Prx7 and Prx8 were introduced by particle bombardment, along with a marker gene encoding Green Fluorescent Protein to identify individual transfected cells *in vivo*. Subsequent development of *Bgh* on transfected barley leaf epidermal cells was studied and compared to control cells transfected with a marker gene encoding ß-Glucuronidase (Figure 1).

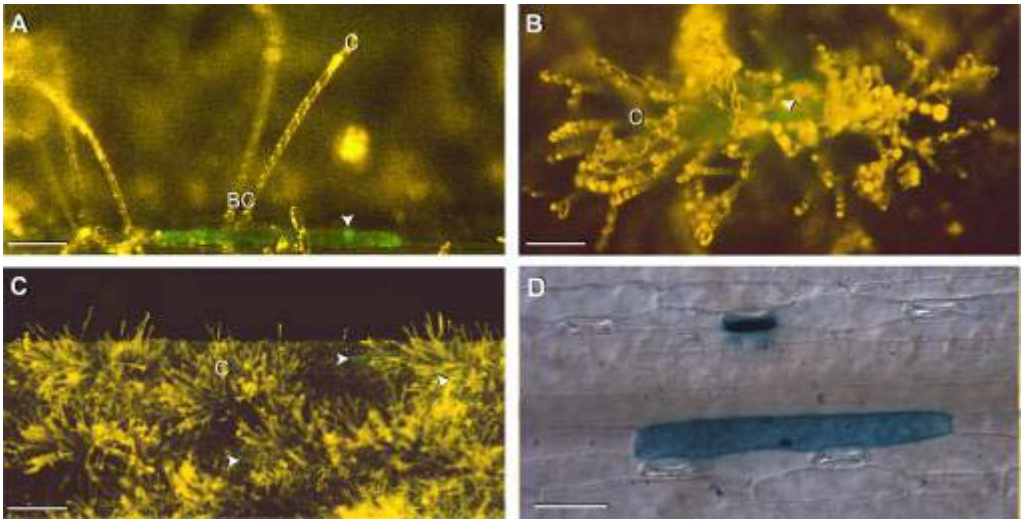


Figure 1. *Transformed barley leaf sections six days after inoculation with* Bgh

A and B: Transformed cells hosting sporulating *Bgh* colonies. C: Overview of the *Bgh* colony density on inoculated leaves. D: Histochemical demonstration of ß-Glucuronidase activity in a detached leaf section three days after transient transformation. Arrowheads: Transformed cells expressing GFP (2A, 2B, 2C); BC: basal cells C: conidia. 2A, 2B and 2D: bar = 50µm. 2C: bar = 500µm. Kristensen, Ammitzbøll, Rasmussen & Nielsen (2001) Mol. Plant Pathol 2: 311-317.

Unexpectedly twice the number of powdery mildew colonies was observed on cells expressing Prx7 as compared to control cells (Figure 2). Mutant and truncated versions of the peroxidases were tested to evaluate the importance of targeting and activity. Introduction of either mutant or truncated versions of Prx7 showed that decreased resistance against *Bgh* was dependent on the presence of the C-terminal signal peptide for correct subcellular targeting, but not affected significantly by mutations in the catalytic centre. By transfecting cells with a construct encoding a GUS protein fused with the Prx7 N- and C-terminal signals it was shown that the susceptible phenotype was not due to distortion of the vacuolar pathway. No impact on *Bgh* performance was observed after introduction of a Prx8 expression or mutant constructs.

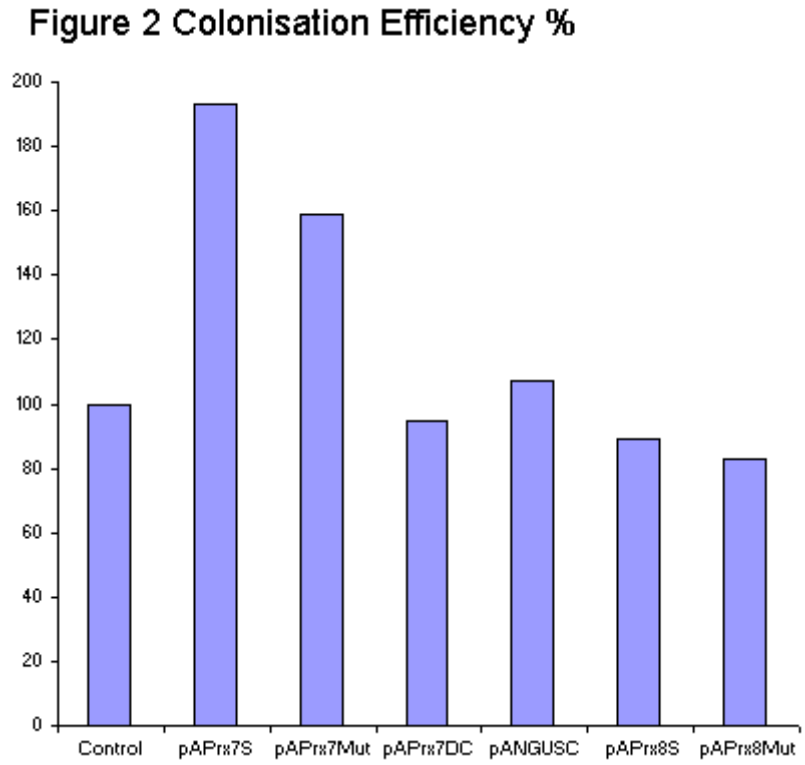


Figure 2. *Relative* Bgh *colonisation efficiency in barley cells transformed with peroxidase constructs.*

Expression constructs: pAPrx7S and pAPrx8S encode native peroxidases. pAPrx7Mut and pAPrx8Mut encode mutant peroxidase variants. pPrx7?C code for the Prx7 peroxidase with the C-terminal signal peptide deleted. pANGUSC encodes a ß-Glucuronidase targeted for the secretory pathway by the Prx7 N- and C-terminal peptides.

These results indicate a more complex role of peroxidases in defence responses than previously suspected. To our knowledge this is the first example of a plant peroxidase that appears to support growth of a pathogen.

Degradation and plant uptake of organic contaminants

Gerda Krog Mortensen
Plant, Soil, and Food Chemistry Programme

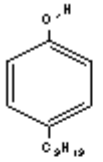
This project has been a part of *Centre for Sustainable Land Use and Management of Contaminants, Carbon and Nitrogen* under The Strategic Environmental Research Programme 1997-2000. The project was finished in 2001.

Recycling of nutrients and organic matter from organic wastes is essential in reducing the need for fertilization and maintaining the soil quality with respect to organic matter content. Therefore, an integral part of developing sustainable agricultural production methods is recycling of wastes. But organic compounds have been measured in high concentrations in waste products such as sewage sludge and therefore, it became essential to reduce contaminant levels and to assess the risks associated with the contaminants introduced in the agro-ecosystem. In Denmark up to 60-70 % of the sewage sludge produced has been used for agricultural purpose.

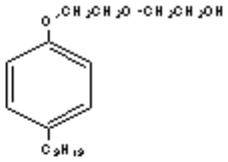
Uptake of chemicals by vegetation is a major source of food chain bioaccumulation and an important route of exposure to humans and animals. Therefore, the plant uptake of chemicals is important in the risk assessment of chemicals.

The contaminants investigated in the project are shown in Table 1.

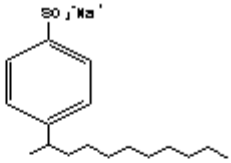
Table 1. Investigated contaminants.



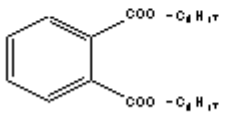
Nonylphenoler
NP



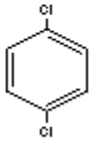
Nonylphenoldiethoxylat
NP EO2



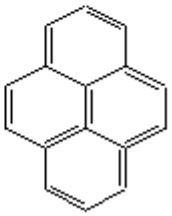
2-C_{12} Lineære alkylbenzensulfonat
LAS



Di(2-ethylhexyl)phthalat
DEHP



1,4-Dichlorbenzen



Pyren

During the project period different experiments have been developed and carried out:

- laboratory experiments, where the metabolization has been investigated in water culture experiments using radiolabeled contaminants
- pot experiments in greenhouse, where degradation and plant uptake were investigated when waste products were incorporated into the soil
- lysimeter experiments, where leaching was investigated
- field experiments, where degradation and uptake were investigated under realistic conditions.

Water culture experiments

The ^{14}C-labeled compounds were spiked into autoclaved and buffered nutrient solution shortly before use. Figure 1 shows the experimental set-up. High adsorption to plant roots

were observed for all the contaminants, although it depended on the plant species and the contaminants.

Figure 1. Rape grown in nutrient solution and radio labeled contaminants

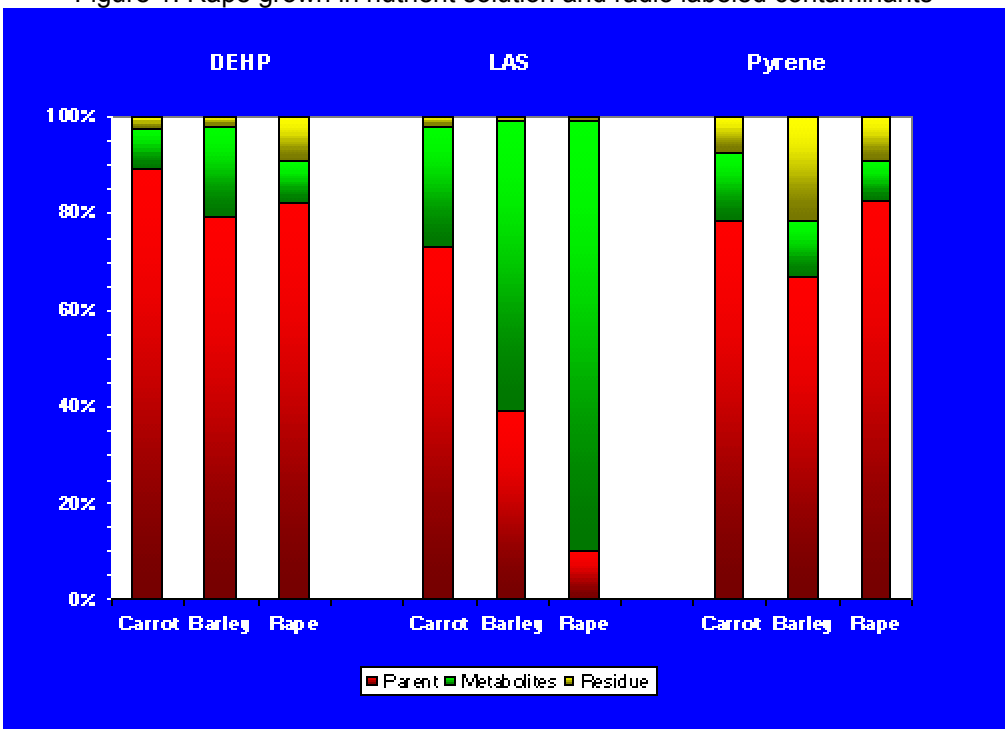


Figure 2. Metabolization of the contaminants.

DEHP and pyrene are very apolar and less soluble in water. Therefore, these contaminants adsorbed strongly to the plant roots and there was nearly no metabolism of these compounds. Las is more water soluble and more metabolization was observed. The results showed the highest metabolization in rape compared to barley and carrots.

Pot experiments.

Figure 3. Rape in sludge amended soil

The waste products used in the pot experiments were:

- anaerobic and aerobic sewage sludge
- compost
- pig manure

The waste products were mixed into the soil but also addition of contaminants as spike solutions were used in some of the experiments. The soil was a sandy soil from Askov, where field experiments were established.

Growth experiments with barley, rape and carrots with growth periods at 20, 30 and 90 days, respectively have been carried out and both planted and plant-free pots were established.

In the experiment with rape anaerobic sludge was added corresponding to 10 t dry matter/ha and the other waste products were added in order to application of the same level of carbon. The degradation of the water-soluble detergent LAS was very fast (Figure 4). After 30 days only 8.7% (2.40 mg kg^{-1} dw in soil) was still found in the soil. When rape was grown in the soil, the degradation of LAS increased, but 5 % (1,36 mg kg^{-1} dw in soil) still remained in the soil. Although the degradation of LAS was quite high, it was not efficient enough to reach the levels of LAS found in uncontaminated soil (< 0.2 mg kg^{-1} dw). In the experiment with carrots with a longer growth period at 90 days, 1-2% still remained in the soil.

In contrast, the degradation of the more hydrophobic plasticiser DEHP was less efficient in soil amended with organic waste products (Figure 4). Apparently, DEHP is more recalcitrant in the soil with degradation of only 18%. Nonylphenol, which is a detergent with estrogenic effects, was degraded faster than DEHP but not as fast as LAS.

Similar to LAS, the growth of rape increased the degradation of DEHP and NP in the treated soil, probably due to either enhanced microbial activity or by aeration of the soil induced by the root growth.

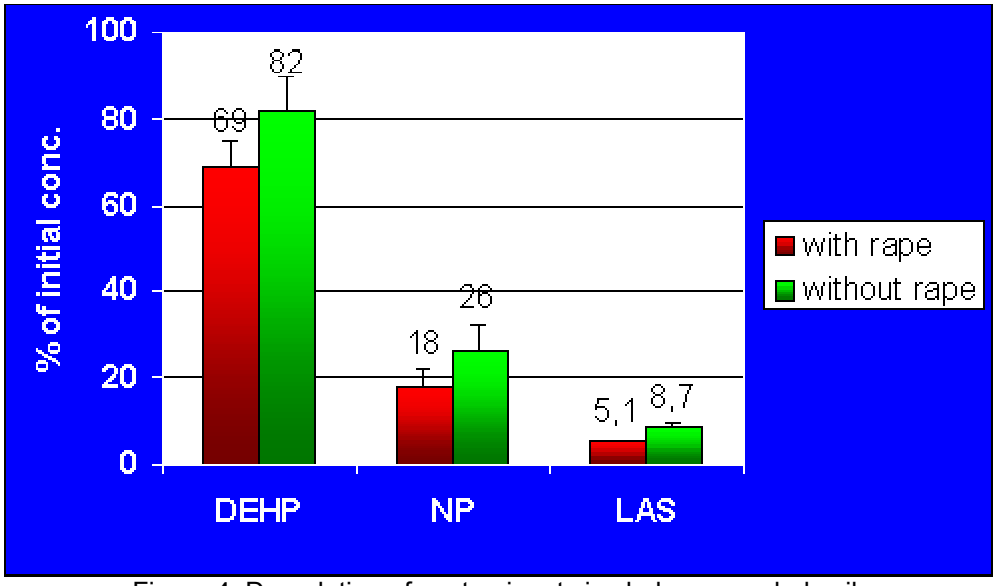


Figure 4. Degadation of contaminants in sludge amended soil.

Lysimeter experiments

Leaching of LAS and NP was investigated in lysimeters using 45 cm soil columns, which were placed outdoor. The soil used was the same as used in the pot experiments. Anerobic sludge was incorporated in the upper soil layer corresponding to 10 t dry matter/ha and barley was sown in the lysimeters. Leachate from the lysimeters and soil samples from 3 layers were collected during the growth period untill harvest of barley.

None of the surfactants were measured above the detection limits in the water samples or in soil layers deeper than 15 cm. The concentrations in the top 15 cm decreased to 25% and 45% of the start concentration for LAS and NP, respectively within the first 10 days. At the end of the study less than 1% and 6% remained. Assuming first order kinetics, half lives at 18 and 33 days were calculated for LAS and NP, respectively. The degradation in the soil is shown in Figure 5.

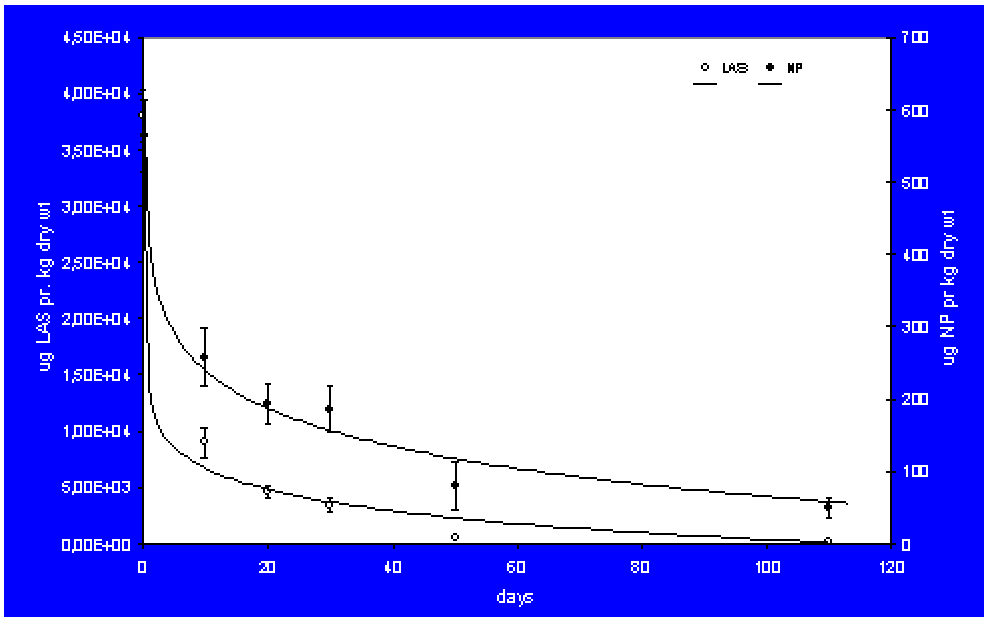


Figure 5. Degradation of LAS and NP in sludge amended soil.

Field experiments

Field experiments were established at Askov experimental station by the *Centre*. Waste products were added in different ways together with experiments, where LAS was spiked to the sludge.

Conclusions

The contaminants were highly adsorbed to plant roots. In water culture experiments there was a potential for plant uptake measured as activity of radiolabeled compounds. But there was a high metabolism of LAS compared to the more apolar contaminants.

The degradation of LAS and NP in soil was fast compared to DEHP. From lysimeter experiments half -lives at 18 and 33 days were calculated for LAS and NP, respectively. In the same experiments no leaching of these compounds were measured. Generally plant growth stimulated the degradation probably due to the higher activity in the soil.

When sludge was applied to soil comparable to general agricultural practice and Danish rules for application, no accumulation in soil from field experiments was observed. And no plant uptake of the investigated contaminants was observed, too. Just in experiments where Las was added to the sludge in very high concentrations, LAS was measured in oat leaves. Concentrations of DEHP in plant leaves were probably due to deposition from the atmosphere.

The use of these different experiments: fast laboratory studies with radiolabeled contaminants, growth experiments in green houses and experiments in the field are very important and useful to investigate the risk of contaminants in the environment.

Fungal spore dispersal

Hanne Østergård
Resistance Biology Programme

Description of spore dispersal patterns is important for predictions for short and long distance spread of diseases. Very different patterns are expected depending on the dispersal function. A central point for much discussion has been whether spore dispersal from a single point source is best described by an exponentially decreasing density, or by a more thick-tailed distribution like a power law density (reciprocal of a polynomial). In the latter case more spores are dispersed over a long distance. We have studied this problem using different stochastic models as well as experimental data.

A spatially-explicit, individual based stochastic simulation model has among others been used for comparing the spatial pattern resulting from different dispersal functions (cf. Lett and Østergård, 2000). The difference in pattern between the dispersal from a point source (focal dispersal) when spores are dispersed according to an exponential function or a power law (in this case, a Cauchy function) with the same median flight distance is shown in Figure 1. The latter pattern (Figure 1A) corresponds to what is observed for light wind-dispersed spores like those of the wheat yellow rust fungus, *Puccinia striiformis* f.sp. *tritici* (see Figure 2).

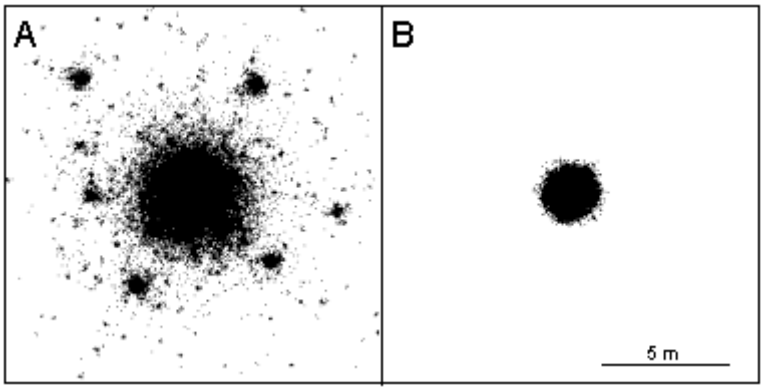


Figure 1. Simulated spatial distribution of the incidence of diseased plants with (A) a Cauchy distribution and (B) an exponential distribution with equal median and no wind effect. Each black dot is a diseased plant. Each white dot is a healthy plant. The epidemic started from four infections in the centre. The pattern shown was obtained 80 days after inoculation using parameters from yellow rust disease on a susceptible wheat cultivar.

Figure 2. Aerial photo of a yellow rust disease focus in a susceptible wheat cultivar plot (9 x 9 m^2) about 50 days after inoculation. (H. Goyeau & C. de Vallavieille-Pope, INRA,Grignon, France)

A theoretical background for understanding the two types of dispersal modes from a point source as essentially the same phenomenon has been one of the results of the work on dispersal of spores going on in our group (Stockmarr, 2002) in collaboration with Viggo Andreasen, Roskilde University. By assuming that spores are released at a certain height about the ground and deposited when they reach a specified lower level, a new stochastic model was derived which combines a new description of spore deposition with a standard stochastic diffusion process in three dimensions. The parameter values for gravitation and wind decide the mode of dispersal. Dispersal, in case of absence of wind, is non-exponential if and only if gravitation is ignorable compared to movement due to diffusion, *i.e.* the spores are very light. However, if non-ignorable constant wind is present, the dispersal along a line transect follows a power law only in the downwind direction. In all other directions the dispersal is exponential. These predictions are supported by available published data indicating that dispersal of small spores (e.g. of size less than 10 μm) is, in general, better described by a power law than by an exponentially decreasing density. The model has been used in a study by Karsten Bjerre of dispersal of the wheat yellow rust fungus; this in collaboration with Mogens Hovmøller, Danish Institute of Agricultural Sciences, and Lisa Munk, The Veterinary and Agricultural University. As yellow rust shows a focal dispersal pattern in the field, disease development of yellow rust can experimentally be modelled by dispersal of spores from a point source. Three experiments have been performed in two years at two locations (Risø and Flakkebjerg) where the spore source consisted of pots of wheat plants with sporulating lesions placed in the center of plots of size 8 x 8 m^2, subdivided into a number of observation cells of size between 0.25 m^2 and 4 m^2 (Figure 3).

Figure 3. Introduced pot with wheat plants having sporulating yellow rust lesions from which spores are spread to the surrounding field plants.

Dispersal of spores from the point source was assessed by evaluating diseased leaf area in each cell before the end of twice the latent period. Climatic data were available at the two locations for the specific period. The observed dispersal patterns revealed a strong influence of climate including wind movement of spores as well as influence of temperature and humidity on spore release, deposition and infection establishment. The dispersal of spores in the different years and locations were according to a thick-tailed dispersal function with a clear influence of wind in one or two directions. The model described above fitted the data within reasonable limits (Figure 4) and gave a better fit than two other stochastic diffusion models with other rules for spore release and deposition.

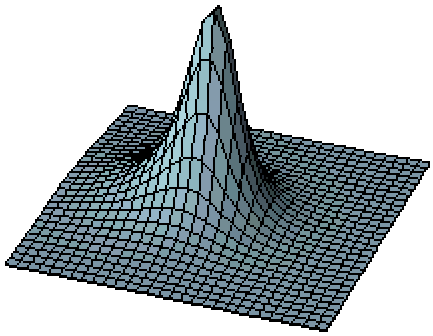


Figure 4. Estimated spore dispersal curve close to the spore source based on the Stockmarr-model for field data from May 17-23, 2001. The spore source was present from April 24-30, 2001. Grid size on figure is 5 cm, i.e*. the figure represents a square of 1.45 x 1.45 m^2.*

The different models are applied for further analysis of experiments in Flakkebjerg of disease development of yellow rust in variety mixtures.

References:

- Lett C., Østergård H. (2000) A stochastic model simulating the spatiotemporal dynamics of yellow rust on wheat. **Acta Phytopathologica et Entomologica Hungarica** 35, 287-293.
- Stockmarr A. (2002) The distribution of particles in the plane dispersed by a simple 3-dimensional diffusion process. **Journal of Mathematical Biology** (*In press*).

Resistance in winter wheat to septoria tritici blotch

Lars Eriksen, Michael F. Lyngkjær and Solveig K. Christiansen
Resistance Biology Programme

Septoria tritici blotch is caused by the fungus *Mycosphaerella graminicola*. Over the past decade this disease has become one of the most important diseases of wheat in Northern Europe. In the field, farmers control septoria tritici blotch by the application of fungicides. However, there is an increasing demand for the newly developed resistant winter wheat cultivars that has appeared in recent years. In the Resistance Biology Programme we focus on the genetic control of resistance to septoria tritici blotch and on the mechanisms of resistance at the cellular level.

Genetic control of resistance to septoria tritici blotch in the cultivar Senat
In a postdoctoral project in collaboration with Sejet Plantbreeding, the inheritance of resistance in field and growth chamber of the winter wheat cultivar Senat is investigated. The purpose of the work is to develop molecular markers, which can be used for the selection of resistant plants in a commercial breeding program. One resistance gene has been located on chromosome 3A (Figure 1). This gene provides complete resistance to the *M. graminicola* isolate IPO323 (provided by G.H.J. Kema) in growth chamber, and in addition it appears to be one of two genes needed for resistance to the isolate Risø97-86. The microsatellite marker *Xgwm369* is closely linked to the resistance gene and can be used for selection in breeding programs. In an artificially inoculated field experiment at Sejet Plantbreeding the resistance gene locus acted as a QTL. A preliminary QTL analysis showed a significant effect of the area on chromosome 3A where the resistance gene is located (Figure 1). The work continues in 2002 with the aim of generating a complete molecular map with microsatellite and AFLP markers allowing a full QTL analysis.

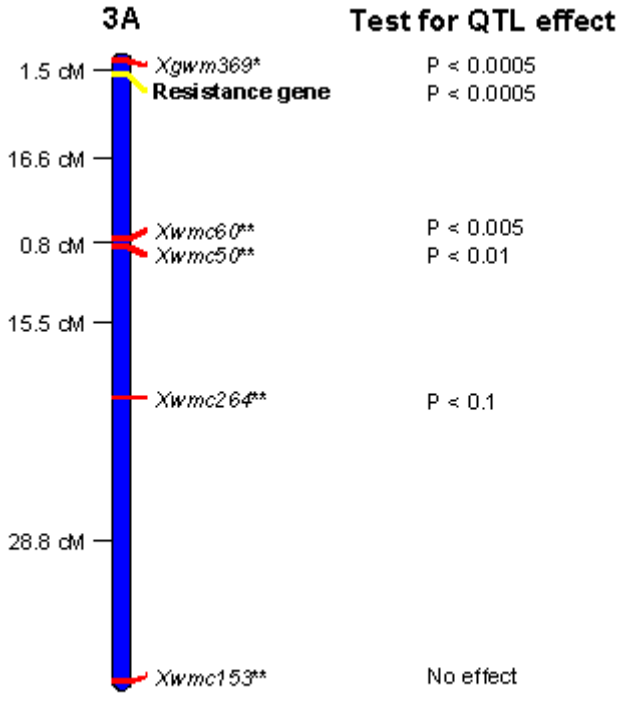


Figure 1. Linkage map of wheat chromosome 3A showing the positions of microsatellite markers and a resistance gene to M. graminicola*. To the right is shown the test for QTL-effect of the six loci in a field experiment.*

Studies on fungal growth in the wheat leaf

Very little is known about the mechanisms that cause resistance against *M. graminicola* in wheat and what determines fungal virulence and avirulence. To clarify this, a study of fungal infection biology and mechanisms of resistance in wheat was initiated. Because *M. graminicola* grows mainly intercellularly in the leaf, it was difficult to make microscope assessment of fungal growth *in planta* using standard histobotanical methods. Therefore, we have modified different *M. graminicola* isolates to express GFP (green fluorescence protein) as a marker to visualise the fungus. By confocal microscopy it was then possible to follow and compare compatible (Figure 2) and incompatible attempted infection *in vivo* on the wheat cultivar Stakado. The results will be published in 2002.

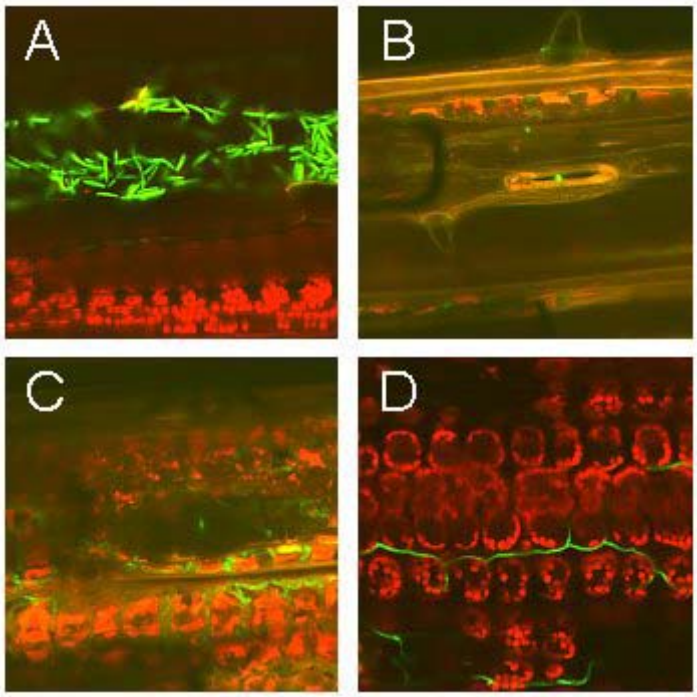


Figure 2. Optical sections from confocal microscopy of compatible interaction between GFP (green fluorescence protein) expressing M. graminicola *and the wheat cultivar Stakado, 18 days after inoculation. A) Fungal spores on the surface of a wheat leaf. B) Fungal hyphae growing through a leaf stoma. C) Fungal hyphae growing between mesophyll cells just below the stoma sub-cavity D) Hyphal growth between mesophyll cells further down in the leaf.*

Seven avirulence genes mapped in the genome of the powdery mildew fungus

Carsten Pedersen and Anna Holefors
Resistance Biology Programme

Research on the powdery mildew disease on barley has a long tradition at Risø with emphasis both on understanding resistance mechanisms of the plant and virulence of the causal agent, the powdery mildew fungus, *Blumeria graminis*. A large number of powdery mildew resistance genes have been identified in barley and used intensively in barley breeding. Unfortunately, these race specific resistances breaks down after relative few years due to increase in frequency of new virulence in the powdery mildew fungus populations. A better understanding of the molecular mechanisms underlying resistance and virulence is hoped to provide the basis for developing strategies for more durable resistance. Recently, the first alleles of the *Mla*-resistance gene locus has been cloned and characterised, but it is still not known how the products of the resistance genes perceive the signal from the invading fungus. According the gene-for-gene model there is an avirulence gene corresponding to each resistance gene and it is speculated that the products of avirulence genes directly or indirectly interact with the resistance gene products as in an elicitor-receptor model. We can therefore predict that the *B. graminis* has a large number of avirulence genes, but none of these have yet been cloned. Isolation and characterised of avirulence genes of the powdery mildew fungus will allow us to study recognition and activation of resistance at the molecular level.

We have now developed a high-density genetic map based on segregation in 81 progeny isolates using mainly AFLP- and RFLP-markers and seven avirulence genes. The map is made up of 34 linkage groups and covers about 2,100 cM totally (Pedersen *et al*., 2002). Here we show just one of the linkage groups (Figure 1). It is assumed that the fungus has about 10-12 chromosomes, so many of the smaller linkage groups are expected to be located on the same chromosomes. However, it will require a significantly larger mapping population and more markers to connect these linkage groups. The map contains 359 markers including the seven avirulence genes. Among the RFLP-markers there are 99 sequenced cDNA-clones, so-called ESTs, so we can assign function to a number of the markers on the map.

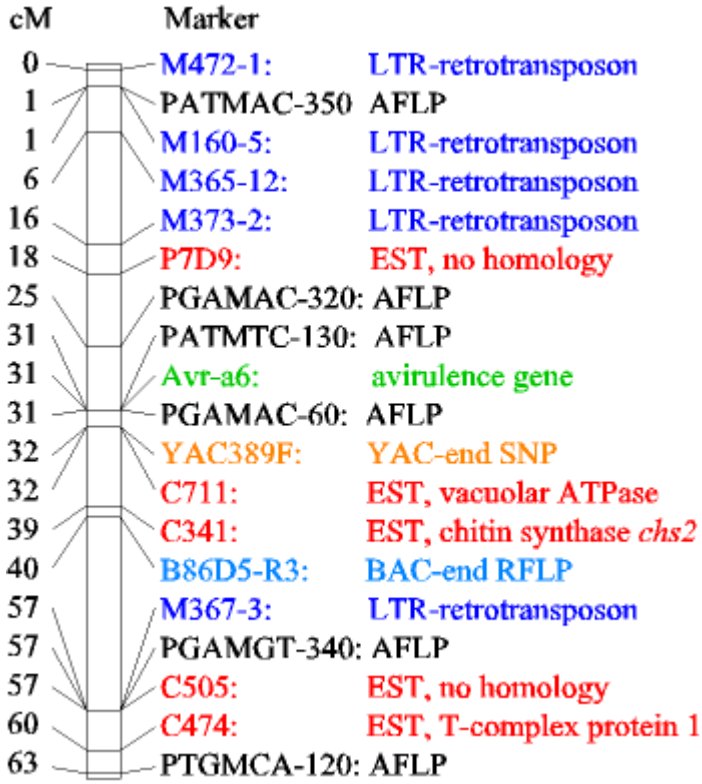


Figure 1. *A linkage group of the genetic map of B. graminis. Genetic distances in centiMorgan (cM). The avirulence gene Avr$_{a6}$ is located in the middle of the group. The markers are coloured according to marker-type.*

The genetic map serves several purposes. The main aim has been to map avirulence genes and identify tightly linked markers for map-based cloning of avirulence genes. Some of the EST-markers were also mapped to a *B. graminis*-map developed by James Brown and co-workers at John Innes Centre, UK, which means that we are able to integrate these two maps and thereby extend the number of markers in regions of interest. The EST-markers are also of high value if or when a genomic sequencing project is initiated, because they will provide a framework of the genome. Finally, the EST-markers let us study if gene-order is preserved between *B. graminis* and other filamentous fungi.

A *B. graminis* BAC-library (BAC=bacteral artificial chromosome) has been established previously and is now used for making so-called contigs, which are arrays of overlapping BAC clones. We have made BAC-contigs at two avirulence gene regions. One is at the Avr$_{a6}$-locus shown on figure 1, and the other is a region composed of three closely linked avirulence genes (Figure 2). We are now focusing on the latter region because we have co-segregation between four markers developed from BAC-ends and an EST-marker. Two BAC-clones spanning this region are now being sequenced and the analysis of the sequence data is in progress. There is a high chance that the avirulence gene Avr$_{a22}$ can be found on these BAC-clones.

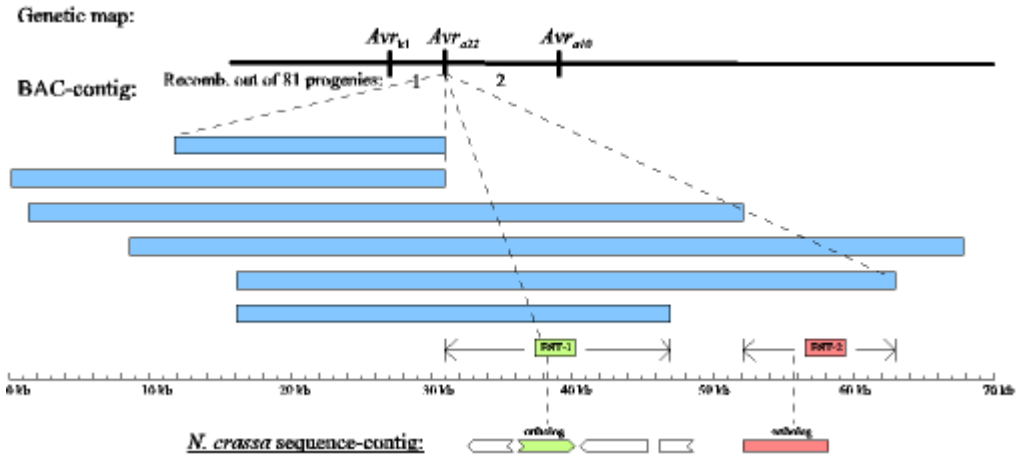


Figure 2. *A region of the B. graminis-genetic map with three avirulence genes (the uppermost part), a BAC-contig made up of six overlapping BAC-clones (in the middle), and the corresponding N. crassa sequence containing the orthologs to the two genes located at BAC-contig (at the bottom).*

One interesting outcome of the EST-based gene map was the demonstration of conserved gene-order between the powdery mildew fungus and two other filamentous fungi, *Neurospora crassa* and *Aspergillus fumigatus*. We studied the cases where we had co-segregating EST-markers and used the sequences of the EST-markers in searching for orthologs in the genomes of *N. crassa* and *A. fumigatus*, which are both fully sequenced and assembled into large contigs. In two out of three well-studied cases micro-synteny could be demonstrated (Figure 3) indicating that synteny at the micro-scale level might be a common phenomenon in fungi as it is among plants and animals. We are now using synteny for expanding BAC-contigs by bridging to *N. crassa* and looking in the corresponding region for putative genes, which are then used for searching the *B. graminis* EST-database. By this approach we used the *B. graminis* EST-1 to identify EST-2, which was found to be located on the same BAC-contig (Figure 2).

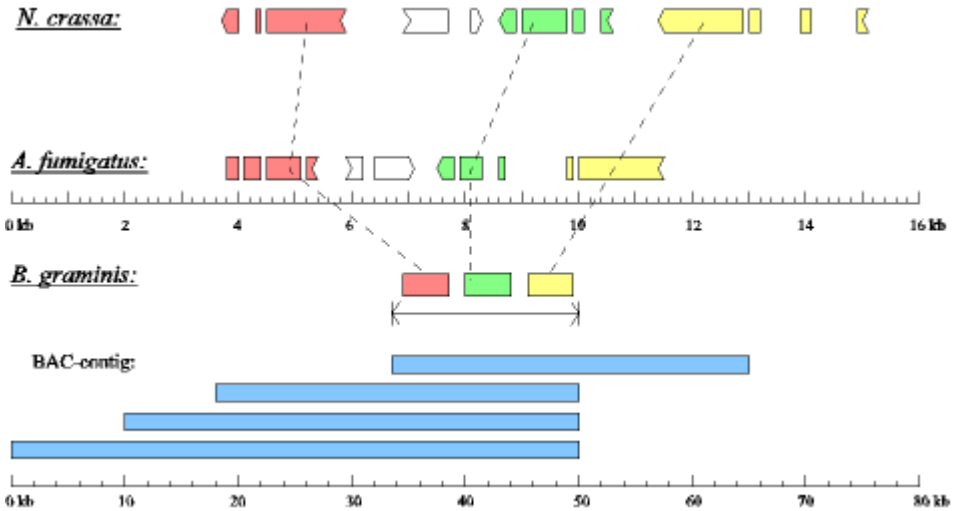


Figure 3. *Micro-synteny between* B. graminis *and the two model-species,* N. crassa *and* A. fumigatus.

Reference:

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Eriksen, Lars B., Sejet Plant Breeding, Denmark (1 year)
Eva Müllerova, Academy of Sciences of the Czech Republic (2 weeks)

Formanek, Pavel, Mendel Agricultural and Forestry University, Czech Republic (8 months)
Gao, Lingling, University of Adelaide, Australia (1 month)
Hindsgaul, Claus, Danish Technical University, Lyngby, Denmark (3 months)
Igamberdiev, Abir, Umeå University, Sweden (8 months)
Naghavi, Reza, Tehran University, Iran (4 months)
Ohura, Mariko, University of Shizuoka, Japan (1 month)
Ohura, Takeo, University of Shizuoka, Japan (1 month)
Roberto Pilu, University of Pavia, Italy (2 weeks)
Sherlock, David John, University of York, GB (2 months)
Søe, Astrid, Max Planck Institute, Germany (1 month)
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Acronyms

ADP	Adenosine Diphosphate
AFLP	Amplified Fragment Length Polymorfism
AGL	Agamous like
AMF	Arbuscular Mycorrhizal Fungi
AP1	Apetala1
ATP	Adenosine Triphosphate
Avr	Avirulence
BAC	Bacterial Artificial Chromosome
Bgh	*Blumeria graminis* f.sp. *hordei*
CONFIRM	Centre for Continuous Flow Isotope Ratio Mass Spectrometry
DEHP	Di(2-ethylhexyl)phthalat
EST	Expressed Sequence Tag
EXOTIC	Exon trapping Consortium
GFP	Green Fluorescent Protein
GUS	Beta-glucuronidase (Beta-D-glucuronoside glucuronosohydrolase)
HC	Hyphal Compartment
ID1	Indeterminate 1
IRMS	Isotope Ratio Mass Spectrometry
LAS	Linear Alkylbenzene Sulphonate
LFY	LEAFY
LM	Light Microscopy
LpTFL	Lolium perenne Terminal Flower
LSCM	Laser Scanning Confocal Microscopy
LSU	Large Ribosomal Subunit
MTBE	Methyl-tertiary-butyl-ether
NMR	Nuclear Magnetic Resonance
NP	Nonylphenol
NPEO2	Nonylphenoldiethoxylat
PCR	Polymerase Chain Reaction
ppm	parts per million
PRD	Plant Research Department
QTL	Quantitative Trait Locus
RC	Root Compartment
RERAF	Risø Environmental Risk Assessment Facility
RFLP	Restriction Fragment Length Polymorfism
SHF	Separate Hydrolysis and Fermentation
SSF	Simultaneous Saccharification and Fermentation
TFL	Terminal Flower 1
UidA	= GUS

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